

Nomenclature of the Proteins of Cows' Milk—Sixth Revision

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ABSTRACT

This report of the American Dairy Science Association Committee on the Nomenclature, Classification, and Methodology of Milk Proteins reviews changes in the nomenclature of milk proteins necessitated by recent advances of our knowledge of milk proteins. Identification of major caseins and whey proteins continues to be based upon their primary structures. Nomenclature of the immunoglobulins consistent with new international standards has been developed, and all bovine immunoglobulins have been characterized at the molecular level. Other significant findings related to nomenclature and protein methodology are elucidation of several new genetic variants of the major milk proteins, establishment by sequencing techniques and sequence alignment of the bovine caseins and whey proteins as the reference point for the nomenclature of all homologous milk proteins, completion of crystallographic studies for major whey proteins, and advances in the study of lactoferrin, allowing it to be added to the list of fully characterized milk proteins.

(Key words: milk protein, structure, nomenclature, review)

Abbreviation key: C = constant, EIMS = electrospray ionization MS, H = heavy chain, HSA = human SA, L = light chain, LF = lactoferrin, MS = mass spectroscopy, NMR = nuclear magnetic resonance, SA = serum albumin, V = variable.

INTRODUCTION

The initial report of the American Dairy Science Association Committee on the Nomenclature, Classification, and Methodology of Milk Proteins (Jenness et al., 1956) was an attempt to clarify the nomenclature of milk proteins by “presenting a summary of preferred usage and by showing the relationship between the individual proteins, which had been isolated, and the classical fractions.” Subsequently, this Committee has published a revision of milk protein nomenclature approximately every 5 to 10 yr to summarize more recent findings (Table 1) and to suggest changes in nomenclature where appropriate. The intent of this Committee is to suggest a flexible nomenclature system that allows for incorporation of new discoveries rather than to suggest prematurely a rigid system of nomenclature. Since the last report of this Committee (Eigel et al., 1984), the most significant findings related to nomenclature and protein methodology are as follows.

- Elucidation of the primary structures of many proteins associated with the fat globule membrane

The rapid progress in this latter area and the importance of these membrane-associated proteins to milk led to a separate review of these proteins. In a review sponsored by this Committee, the current nomenclature for proteins associated with the milk fat globule membrane has been summarized (Mather, 2000). This report will deal only with the proteins of skim milk. In addition, in keeping with past practice, we will not elaborate on enzymes associated with whole milk or skim milk. These enzymes have been reviewed elsewhere (Shahani et al., 1973; Jenness, 1974; Swaisgood, 1995).

- New genetic variants and milk proteins

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Table 1. Proteins of bovine milk and some of their properties.¹

Protein (suggested abbreviation)	Composition in skim milk (g/L)	Genetic variants ²	Molecular weight ³	Isoionic point ⁴	Isoelectric point ⁵	A ¹ % 1 cm ⁵	H _φ Ave ⁶ (kcal/res.)
α _{s1} -Casein (α _{s1} -CN)	12–15	B	23,615	4.92–5.05	4.44–4.76	10.05	1170
		C	23,542	5.00–5.35	...	10.03	1170
α _{s2} -Casein (α _{s2} -CN)	3–4	A	25,226	1111
β-Casein (β -CN)	9–11	A ¹	24,023	5.41	1322
		A ²	23,983	5.30	4.83–5.07	4.6, 4.7	1335
		B	24,092	5.53	—	4.7	1326
κ-Casein (κ -CN)	2–4	A	19,037	5.77 (5.35)	5.45–5.77	—	1205
		B	19,006	6.07 (5.37)	5.3–5.8	10.5	1224
β-Lactoglobulin (β -LG)	2–4	A	18,363	5.35	5.13	9.6	1211
		B	18,277	5.41	5.13	10.0, 9.6	1217
α-Lactalbumin (α -LA)	0.6–1.7	B	14,178	—	4.2–4.5	20.1–20.9	1120
Serum albumin (SA)	0.4	A	66,399	5.13	4.7–4.9	6.3–6.9	1120
Immunoglobulin G1 (IgG1)	0.3–0.6	...	161,000	—	5.5–6.8	13.6	...
Immunoglobulin G2 (IgG2)	0.05	...	150,000	—	7.5–8.3	13.6	...
Immunoglobulin A ⁷ (IgA)	0.01	...	385,000–417,000	12.1	...
Immunoglobulin M (IgM)	0.09	...	1,000,000	12.1	...
Secretory component (SC)	0.02–0.1	...	63,750	7.48	...	15.5	...
Lactoferrin (LF)	0.02–0.1	...	76,110	8.95	8.81	9.91	1053

¹Inclusion of values in this table does not constitute endorsement by the Committee.

²Major variants.

³Calculated as formula weight (3 decimal places) from composition. All acidic groups are protonated, all basic groups are not protonated. Where known, the major disulfide linkages are taken into account; κ-CN has no disulfides, but its N-terminal pyroglutamic is included. Immunoglobulins represent a range.

⁴Eigel et al. (1984).

⁵Absorptivity of a 1% solution measured in a 1-cm light path at 280 nm, except where noted otherwise.

⁶Average hydrophobicity calculated using free energies of transfer of amino acid side chains from an organic environment to an aqueous environment composition of the various proteins (Bigelow, 1967).

⁷All physical and chemical data were obtained with secretory IgA. More details on all Ig are given in Table 5.

The discovery of new genetic variants has contributed to the establishment of a broader base of information on milk proteins (Formaggioni et al., 1999; Ng-Kwai-Hang and Grosclaude, 2003). The widespread application of molecular biology techniques to probe the “milk protein genome” has brought about an interesting dilemma in the study of milk proteins. Because of the built-in redundancy in the genetic code, it is possible to have a multiplicity of, e.g., κ-CN genes *CSN3*, that will code for the same protein (κ-CN). Note that the designation at the *CSN3* locus relates to the DNA sequence or genotype, and CN denotes the phenotypic expression of the gene, the protein molecule. For example, Prinzenberg et al. (1999) demonstrated the presence of a silent mutation in the *CSN3**A locus; here, the change of CCA to CCG still codes for proline at residue 152. This locus is termed *CSN3**A₁, but the protein remains κ-CN A. As an interesting aside, for single codon changes in the 3 base code, there could be up to 575 silent changes in the DNA, yet the expressed protein would remain κ-CN A. Because this Committee has focused on protein methodologies and sequences, it is the intent to assign letter symbols only to those proteins that are chem-

ically different from the reference protein or a known member of the family. In this respect, no matter how many codon changes there are in the DNA, if the protein is chemically identical to a member of the family, then it would still be, e.g., κ-CN A.

- Establishment by sequencing techniques and sequence alignment of the bovine CN and whey proteins as the reference point for the nomenclature of all homologous milk proteins. In this regard, several free on-line protein databases have been established.
- In addition to serving as on-going information repositories, these web sites contain convenient programs for the calculation of theoretical protein parameters, sequence alignments, and structural predictions. The following list contains some of these sites, but others are equally valuable.
 - National Center of Biotechnology Information: www.ncbi.nih.gov/database
 - The Swiss Protein and European Molecular Biology Site (ExPASy): www.expasy.org
 - Georgetown University Protein Information Resource: <http://pir.georgetown.edu>

- Protein Science Site: <http://www.proteinsociety.org/>
- A new database for protein researchers is being constructed under an NIH contract. It will combine the current Swiss-Prot, Trémbl, and PIR resources into a single searchable database. The new resources will be called United Protein Data Base or UniProt, and, when completed (~2005), its web site will be www.uniprot.org. The current sites will continue to function as conduits for the new database.
- The European Bioinformatics Institute: www.ebi.ac.uk
- Comparative Ig Web site: www.medicine.uiowa.edu/CIgW
- IMGT Ig web site: <http://imgt.cines.fr/>
- Characterization of all bovine Ig at the molecular levels, including mapping of the heavy chain (H) constant (C) region locus, defining the mechanism of variable heavy- and lambda- chain repertoire development, and establishing international standards for nomenclature (www.medicine.uiowa.edu/CIgW).
- Crystallographic studies have been completed on the major whey proteins as detailed subsequently. Crystal structures for viewing are available through the Protein Data Bank at www.rcsb.org/pdb. In the absence of crystallographic data for the CN, working 3-D molecular models have been presented as a point of departure for future studies of CN structure (Kumosinski et al., 1994). These models are available at www.arserrc.gov/dpp/casein.htm.
- Progress in the sequence analysis of lactoferrin (LF) allows this protein to be reviewed for the first time by this Committee.

THE CASEINS

Caseins in milk of the genus *Bos* were defined originally by this Committee (Jenness et al., 1956) as those phosphoproteins that precipitate from raw skim milk by acidification to pH 4.6 at 20°C. In a subsequent report (Whitney et al., 1976), the Committee differentiated CN according to their relative electrophoretic mobility in alkaline polyacrylamide or starch gels containing urea with or without mercaptoethanol. In the previous report, we recommended that use of electrophoresis as a basis for classification be dropped and that CN be identified according to the homology of their primary structures (amino acid sequences) into the following families: α_{S1} -, α_{S2} -, β -, and κ -CN. This recommendation is affirmed, and researchers are requested to refrain from assigning specific genetic variant letters

to new variants until their sequence homology can be established. Individual members of these families still can be identified by gel electrophoretic techniques, some of the more effective of which are suggested in the monograph by this Committee (Swaigood et al., 1975).

α_{S1} -CN

The α_{S1} -CN family, which constitutes up to 40% of the CN fraction in bovine milk, consists of one major and one minor component. Both proteins are single-chain polypeptides with the same amino acid sequence established by Mercier et al. (1971) and Grosclaude et al. (1973) and differ only in their degree of phosphorylation. The minor component contains one additional phosphorylated serine residue at position 41 (Eigel et al., 1984). The reference protein for this family is α_{S1} -CN B-8P, a single-chain protein with no cysteinyl residues. It consists of 199 amino acid residues: Asp₇, Asn₈, Thr₅, Ser₈, Ser P₈, Glu₂₅, Gln₁₄, Pro₁₇, Gly₉, Ala₉, Val₁₁, Met₅, Ile₁₁, Leu₁₇, Tyr₁₀, Phe₈, Lys₁₄, His₅, Trp₂, and Arg₆ with a calculated molecular weight of 23,615 (Mercier et al., 1971). Its primary sequence is given in Figure 1; its ExPASy entry name and file number are CAS1_Bovine and P02662, respectively. Since the last nomenclature report (Eigel et al., 1984), 3 new genetic variants of α_{S1} -CN have been identified. They are α_{S1} -CN F (Erhardt, 1993), which was found in German Black and White cattle; α_{S1} -CN G (Mariani et al., 1995) discovered in Italian Brown cows; and the H variant (Mahé et al. 1999). Hence, this family of proteins is currently known to consist of variant A found in Holstein Friesians, Red Holsteins, and German Red cattle (Ng-Kwai-Hang et al., 1984; Grosclaude, 1988; Erhardt, 1993); variant B, which is the predominant variant in *Bos taurus* (Eigel et al., 1984); variant C in *Bos indicus* and *Bos grunniens* (Eigel et al., 1984); variant D in various breeds in France (Grosclaude, 1988) and Italy (Mariani and Russo, 1975) as well as in Jerseys in The Netherlands (Corradini, 1969); and variant E in *Bos grunniens* (Grosclaude et al., 1976) in addition to the new variants F, G, and H.

The primary structure of α_{S1} -CN B is given in Figure 1, and the deletion or substitutions for its genetic variants are given in Table 2. The structure of α_{S1} -CN B was determined by amino acid sequencing (Mercier et al., 1971; Grosclaude et al., 1973) and confirmed by cDNA sequencing (Nagao et al., 1984; Stewart et al., 1984) and by sequencing of the genomic DNA (Koczan et al., 1991). The α_{S1} -CN signal peptide is composed of 15 amino acid residues, making the pre-form of α_{S1} -CN B 214 amino acids in length. Variant A uniquely arises as a result of exon skipping caused by a single-base mutation that affects splicing of the pre-mRNA (Mohr

1	10	20
H-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-		
21	30	40
Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-		
41	50	60
<u>Ser</u> -Lys-Asp-Ile-Gly- SeP -Glu- SeP -Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met-		
61	70	80
Glu-Ala-Glu- SeP -Ile- SeP - SeP - SeP -Glu-Glu-Ile-Val-Pro-Asn- SeP -Val-Glu-Gln-Lys-His-		
81	90	100
Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-		
101	110	120
Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn- SeP -Ala-Glu-Glu-Arg-Leu-		
121	130	140
His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-		
141	150	160
Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-		
161	170	180
Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-		
181	190	199
Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-OH		

Figure 1. The primary structure of α_{S1} -CN B-8P (Mercier et al., 1971; Grosclaude et al., 1973; Stewart et al., 1984; Nagao et al., 1984; Koczan et al., 1991). Amino acid deletion or substitutions for genetic variants A, C, D, E, F, G, and H, respectively, are indicated in Table 2. Sites of post-translational phosphorylation (*SeP*) are indicated in italicized, boldface type. The underline indicates the location of another phosphorylation site in a minor species of this protein (α_{S1} -CN B9-P).

et al., 1994). Deletion of residues 14 through 26 was first identified by amino acid sequencing (Grosclaude et al., 1970) and later confirmed by cDNA sequencing (McKnight et al., 1989). Neither the primary structures nor nucleic acid sequences of variants F and G have been completely reported.

The secondary structure of α_{S1} -CN has been examined by various methods including CD spectroscopy, Raman spectroscopy, and predictive algorithms using sequence information, and the results have been reviewed previously (Swaigood, 1992). However, its 3-D structure cannot be determined because the protein does not form crystals. Nuclear magnetic resonance (NMR) studies have also proven to be problematic because of the intrinsic aggregation of the protein. Nevertheless, its tertiary structure has been predicted using a combination of predicted secondary structures, adjusted to conform to the amount of global secondary structures determined experimentally, with molecular-modeling computations based on energy minimization (Kumonsinski et al., 1994). The latter structure should be viewed as a working model, which is consistent with bulk properties of the protein; it represents one possible interpretation of its structure.

Since the discovery of genetic variants, attempts have been made to correlate milk characteristics or milk production with the genotype. However, the correlations obtained have not been straightforward, in part because of differences in the parameters used. For example, the

α_{S1} -CN BB phenotype has been correlated with higher milk yields and, thus, higher protein yield over the lactation, (Ng-Kwai-Hang et al., 1984; Aleandri et al., 1990; Sang et al., 1994), but the same phenotype has also been correlated with lower protein concentration in milk (Ng-Kwai-Hang et al., 1986, 1992). It appears that cows carrying the G allele produce less α_{S1} -CN and more of the other caseins (Mariani et al., 1995). For example, homozygous (GG) cows produce 55% less α_{S1} -CN.

Because of the 13-amino acid residue deletion, the A variant's characteristics are most different from the other variants (Farrell et al., 1988). Thus, most of the hydrophobic residues in the N-terminal region are eliminated, including the Phe-Phe-Val sequence that is cleaved by chymosin during cheese ripening (Mulvihill and Fox, 1979). Hence, α_{S1} -CN A is similar to the peptide α_{S1} -I corresponding to α_{S1} -CN (f25-199), which has a reduced hydrophobicity (Creamer et al., 1982) and does not aggregate as extensively in the presence of calcium (Kaminogawa et al., 1980). The changes in curd rheology that occur with this proteolysis of the B variant are consistent with the observation that soft curds are formed with milks containing α_{S1} -CN A (Sadler et al., 1968).

Comparison of the properties of variants B and C has indicated that α_{S1} -CN C self-associates more strongly (Schmidt, 1970; Swaigood, 1973), and cheeses made

Table 2. Positions and amino acid differences in genetic variants of milk proteins.

Protein ¹	Variant	Position and amino acid in the protein														
α_{s1} -CN (199)	A	14–26					53	51–58	59		66	192				
	B	Deleted					<i>Ala</i>		<i>Gln</i>		<i>SerP</i>	<i>Glu</i>				
	C											<i>Glu</i>				
	D						ThrP					<i>Gly</i>				
	E								Lys			<i>Glu</i>				
	F											<i>Gly</i>				
	G										Leu	<i>Glu</i>				
	H							Deleted				<i>Glu</i>				
α_{s2} -CN (207)	A	33		47				51–59			130					
	B	<i>Glu</i>		<i>Ala</i>							<i>Thr</i>					
	C	Complete	sequence	not yet	determined											
	D	Gly		Thr				Deleted			Ile					
β -CN (209)	A ¹	18	25	35	36	37	67	72	88	93	106	122	137/138	152	?	
	A ²	<i>SerP</i>	<i>Arg</i>	<i>SerP</i>	<i>Glu</i>	<i>Glu</i>	His	<i>Gln</i>	<i>Leu</i>	<i>Met</i>	His	<i>Ser</i>	<i>Leu/Pro</i>	<i>Pro</i>	<i>Gln</i>	
	A ³						Pro				Gln					
	B						His					Arg				
	C			Ser		Lys	His									
	D	Lys														
	E															
	F				Lys											
	G						His									
	H ¹						His									
	H ²															
	I															
κ -CN (169)	A		10			97	104	135	136			148		155		
	B		<i>Arg</i>			<i>Arg</i>	<i>Ser</i>	<i>Thr</i>	<i>Thr</i>			<i>Asp</i>		<i>Ser</i>		
	C					His			Ile			<i>Ala</i>				
	E															
	F ¹															
	F ²															
	G ¹															
	G ²					Cys										
	H															
	I															
	J						Ala									
β -LG (162)	A	45	50	56	59	64	70	78	108	118	126	129	158			
	B	<i>Glu</i>	<i>Pro</i>	<i>Ile</i>	<i>Gln</i>	<i>Asp</i>	<i>Lys</i>	<i>Ile</i>	<i>Glu</i>	<i>Val</i>	<i>Pro</i>	<i>Asp</i>	<i>Glu</i>			
	C				His	<i>Gly</i>				<i>Ala</i>						
	D	Gln														
	E															
	F		Ser													
	G															
	H						Asp	Asn	Met							
	I															
	J								Gly	Val						
	W										Leu					
α -LA (123)	A					10										
	B					Gln										
	C					<i>Arg</i>										

¹Numbers in parentheses indicate the total number of amino acid residues in the reference protein. Amino acids in the reference protein given in the sequence figures are in *italics*. Genetic variant positions are as indicated for the major milk proteins in regular type.

²Possibly 130, the same substitution.

from milks containing the latter form a tougher curd (Sadler et al., 1968).

The distinct regions of anionic clusters and hydrophobicity evident in the primary structure are suggestive of the formation of hydrophobic and polar domains (Swaigood, 1982, 1992) and are consistent with observed physical-chemical properties, such as the strong dependence of association on concentration, pH, ionic strength, and ion binding. The characteristics and significance of calcium ion binding to the anionic clusters are well known, but it has also been found that Zn^{2+} (Singh et al., 1989) and Fe (III) (Reddy and Mahoney, 1991) bind at these sites. The effect of these interactions on micelle structure and stability is not known.

α_{S2} -CN

The α_{S2} -CN family, which constitutes up to 10% of the CN fraction in bovine milk, consists of 2 major and several minor components exhibiting varying levels of post-translational phosphorylation (Swaigood, 1992) and minor degrees of intermolecular disulfide bonding (Rasmussen et al., 1992). The predominant forms in bovine milk contain an intramolecular disulfide bond and differ only in their degree of phosphorylation. The reference protein for this family is α_{S2} -CN A-11P, a single-chain polypeptide with an internal disulfide bond. It consists of 207 amino acid residues: Asp₄, Asn₁₄, Thr₁₅, Ser₆, Ser P₁₁, Glu₂₄, Gln₁₆, Pro₁₀, Gly₂, Ala₈, Cys₂, Val₁₄, Met₄, Ile₁₁, Leu₁₃, Tyr₁₂, Phe₆, Lys₂₄, His₃, Trp₂, and Arg₆ with a calculated formula molecular weight of 25,226. The primary structure of this protein is given in Figure 2; its ExPASy entry name and file number are CAS2_Bovin and P02663, respectively. The secondary structure of α_{S2} -CN has recently been studied by CD and FTIR spectroscopies (Hoagland et al., 2001).

The genetic variants, identified in the fifth revision of the Nomenclature report (Eigel et al., 1984), are α_{S2} -CN A, B, C, and D. Upon alkaline urea-gel electrophoresis, these proteins migrate between the α_{S1} - and β -CN, and the most prevalent species, α_{S2} -CN A-11P, has served as the reference band for all proteins in the casein pattern (Whitney et al., 1976). The A variant is most frequently observed in Western breeds, with α_{S2} -CN D observed with frequencies of 0.01 to 0.09 in Vosgienne and Montbeliarde breeds (Grosclaude et al., 1978) and in 3 Spanish breeds (Osta et al., 1995b). The B variant was observed with low frequencies in zebu cattle in South Africa and, variant C was observed in yaks in the Nepalese valley and the Republic of Mongolia (Grosclaude et al., 1976, 1982).

The primary structure of α_{S2} -CN A-11P (Figure 2), reported by Brignon et al. (1977), has been changed to Gln at position 87 rather than Glu, as indicated by

cDNA sequencing (Stewart et al., 1987) and genomic DNA sequencing (Groenen et al., 1993). The α_{S2} -CN signal peptide is composed of 15 amino acid residues, making the pre-form 222 amino acid residues in length. The D variant differs from α_{S2} -CN A by the deletion of 9 amino acid residues from positions 51 to 59. However, the genomic DNA sequence does not reveal a deletion, but rather a substitution, suggesting that the amino acid sequence deletion is caused by the skipping of exon VIII, a 27-nucleotide sequence that encodes amino acid residues 51 to 59 (Bouniol et al., 1993). As shown in Table 2, the C variant differs from the A variant at positions 33, 47, and 130 (Mahé and Grosclaude, 1982). As the specific sites of mutation resulting in α_{S2} -CN B have not been identified, as shown in Table 2. Because of the progress made on this protein, following the elucidation of its sequence, α_{S2} -CN will be reviewed in more detail in this report. Post-translational phosphorylation, primarily at seryl residues, results in the incorporation of 10 to 13 phosphate moieties. According to the specificity of CN kinase, phosphorylation occurs at Ser/Thr residues in the sequence Ser/Thr-X-Glu/SerP/Asp; however, the sequence SerX-Glu/SerP is heavily favored (Mercier, 1981). Only seryl residues are phosphorylated in α_{S2} -CN A-11P, but Thr-66 was partially phosphorylated in α_{S2} -CN C (Mahe and Grosclaude, 1982). Those residues known to be phosphorylated in α_{S2} -CN A-11P are indicated by boldface italics in the figure. The underlined residues indicate potential sites of phosphorylation suggested by the enzyme specificity. It should be noted that Thr-47 in α_{S2} -CN C is a potential phosphorylation site.

Another post-translational change that occurs with this protein is the formation of disulfide bonds. The 2 cysteinyl residues of this protein participate in both intramolecular and intermolecular disulfide bonds (Rasmussen et al., 1992, 1994). The protein exists predominantly as a monomer (>85%) with a disulfide bond between Cys residues 36 and 40 (Rasmussen et al., 1994) or as a dimer with both parallel and antiparallel disulfide bonds (Rasmussen et al., 1992). Therefore, 2 types of dimers are found: one fraction with residues 36 and 40 in one chain linked to residues 36 and 40, respectively, in the other chain. But, in another fraction, residues 36 and 40 are linked to residues 40 and 36, respectively, in the other chain. These results suggest that the formation of these bonds is not important to any structure required by this protein for its interaction with other CN.

α_{S2} -Casein is the most hydrophilic of all caseins as a result of the 3 clusters of anionic groups composed of phosphoseryl and glutamyl residues. Although relatively hydrophobic, the C-terminal 47 residues carry a net positive charge (about +9.5) at the pH of milk

1	10	20
H-Lys-Asn- <u>Thr</u> -Met-Glu-His-Val- <i>SeP</i> - <i>SeP</i> - <i>SeP</i> -Glu-Glu-Ser-Ile-Ile- <i>SeP</i> -Gln-Glu-Thr-Tyr-		
21	30	40
Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro- <i>SeP</i> -Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-		
41	50	60
Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly- <i>SeP</i> - <i>SeP</i> - <i>SeP</i> -Glu-Glu-		
61	70	80
<i>SeP</i> -Ala-Glu-Val-Ala- <u>Thr</u> -Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-		
81	90	100
Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-		
101	110	120
Gln-Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-		
121	130	140
Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu- <i>SeP</i> - <u>Thr</u> - <i>SeP</i> -Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-		
141	150	160
Met-Glu- <i>SeP</i> -Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu- <u>Thr</u> -Glu-Glu-Glu-Lys-Asn-Arg-		
161	170	180
Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-		
181	190	200
Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-		
201	207	
Ile-Pro-Tyr-Val-Arg-Tyr-Leu-OH		

Figure 2. The primary structure of the α_{S2} -CN A-11P (Brignon et al., 1977; Mahé and Grosclaude, 1982; Stewart et al., 1987; Groenen et al., 1993). Seryl residues (*SeP*) identified as phosphorylated in α_{S2} -CN A-11P are indicated in italicized, boldface type. Residues that have been determined to be partially phosphorylated or that potentially may be phosphorylated according to CN kinase specificity are underlined. Amino acid deletions or substitutions for genetic variants are given in Table 2.

(Swaigood, 1992). On the other hand, the more hydrophilic N-terminal 68 residues contain 2 anionic clusters and exhibit a net charge of about -21 at the prevalent pH of milk. Hence, the primary structure of α_{S2} -CN can be represented by 4 domains: an N-terminal hydrophilic domain with anionic clusters, a central hydrophobic domain, followed by another hydrophilic domain with anionic clusters, and finally a C-terminal positively charged hydrophobic domain (Swaigood, 1992). This structure is consistent with an association behavior that is very dependent on ionic strength (Snoeren et al., 1980). The association appears to be strongest around an ionic strength of 0.2 M, with dissociation occurring in lower salt because of electrostatic repulsion and also in higher salt because of suppression of electrostatic attraction, thus, reflecting the contributions of both hydrophobic interactions and electrostatic attraction.

The number of anionic clusters and the hydrophilic nature is also reflected in calcium-binding properties of α_{S2} -CN. For example, the latter protein is more sensitive to Ca^{2+} than α_{S1} -CN (Toma and Nakai, 1973), with almost complete precipitation occurring in 2 mM Ca^{2+} for α_{S2} -CN at pH 7; whereas, precipitation of α_{S1} -CN requires 6 mM Ca^{2+} (Aoki et al., 1985). These properties also led to a method for fractionation of α_{S2} -CN from other caseins by precipitation from propan-1-ol solutions (Vreeman and van Riel, 1990). Solubility in this

solvent is governed by electrostatic interactions that are most prevalent in α_{S2} -CN.

α_{S2} -Casein appears to be readily susceptible to proteolysis as assessed by the activities of chymosin and plasmin toward the protein. Chymosin activity was observed in the regions of residues 88 to 98 and 164 to 180, but its primary cleavage occurred at Phe 88-Tyr 89 (McSweeney et al., 1994). These 2 regions are, respectively, at the edge of the central hydrophobic domain or in the first part of the cationic hydrophobic C-terminal domain. Plasmin activity released a number of peptides, including the N-terminal 21 to 24 residues of the initial hydrophilic domain containing one of the anionic clusters (Le Bars and Gripon, 1989; Visser et al., 1989). In agreement with plasmin specificity, mostly Lys-X bonds were cleaved at varying rates (Lys residues 21, 24, 149, 150, 181, 188, and 197). In addition to the shorter N-terminal peptides, a major peptide released was α_{S2} -CN (f51-207) (Le Bars and Gripon, 1989). In this regard, it is interesting to note that recently α_{S2} -CN (f65-203) was isolated from milk and shown to have antibacterial activity (Zucht et al., 1995).

β -CN

The β -CN family, which constitutes up to 45% of the casein of bovine milk is quite complex because of the action of the native milk protease plasmin (Eigel et al.,

1	10	20
H-Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu- SeP -Leu- SeP - SeP - SeP -Glu-		
21	30	40
Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln- SeP -Glu-Glu-Gln-Gln-Gln-		
41	50	60
Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-		
61	70	80
Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-		
81	90	100
Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys-Val-Lys-Glu-		
101	110	120
Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-Phe-Thr-		
121	130	140
Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-		
141	150	160
Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-		
161	170	180
Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr-		
181	190	200
Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-		
201	209	
Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH		

Figure 3. Primary structure of *Bos* β -CN A²-5P (Ribadeau-Dumas et al., 1972; Grosclaude et al. 1973). The amino acid residues corresponding to the mutational differences in the genetic variants, A¹, A³, B, C, D, E, F, G, H, and I are indicated in Table 2. Sites of post-translational phosphorylation (*SeP*) are indicated in italicized, boldface type. The arrows indicate the points of attack by plasmin responsible for β -CN fragments (γ -CN and proteose peptones) present in milk.

1984). Plasmin cleavage leads to formation of γ_1 -, γ_2 -, and γ_3 -CN, which are actually fragments of β -CN consisting of residues 29-209, 106-209, and 108-209. In addition, polypeptides previously called proteose peptone components 5, 8-fast, and 8-slow are fragments of β -CN, which represent residues 1-105 or 1-107, 1-28, and 29-105, respectively. The reference protein for this family, β -CN A²-5P is a single-polypeptide chain with no Cys residues containing 209 residues. It consists of Asp₄, Asn₅, Thr₉, Ser₁₁, Ser P₅, Glu₁₉, Gln₂₀, Pro₃₅, Gly₅, Ala₅, Val₁₉, Met₆, Ile₁₀, Leu₂₂, Tyr₄, Phe₉, Lys₁₁, His₅, Trp₁, and Arg₄ with a calculated molecular weight of 23,983. The most common variant used as reference is variant A²; its ExPASy entry name and file number are CASB_Bovin and P02666, respectively. The A² variant has been chemically sequenced (Ribadeau-Dumas et al., 1972) and sequenced from its cDNA (Jimenez-Flores et al., 1987; Stewart et al., 1987) and its gene (Bonsing et al., 1988). The β -CN signal peptide is composed of 15 amino acid residues, making the pre-form 224 amino acids in length.

The sequence shown for β -CN A² in Figure 3 is that as corrected by 2 groups (Yan and Wold, 1984; Carles et al., 1988). It differs from the original sequence (Eigel et al., 1984) in 4 places: Gln117Glu, Pro 137 and Leu 138 are inverted, Glu175Gln, and Gln195Glu. The changes at residues 117 and 175 are confirmed by both groups and by gene sequencing. The inversion of resi-

dues 137 and 138 are not in agreement with cDNA sequencing data (Jimenez-Flores et al., 1987), which is in accordance with the original data. However the Leu-Pro substitution is a one base change, and mutations could occur and not be observed by HPLC-mass spectroscopy (MS) of peptides or by electrophoresis of the proteins. The weight here is, however, given to the 2 independent protein-sequencing reports. In a similar fashion, the change at 195 is not in agreement with the cDNA results, but, in this case, 3 lines of evidence support the occurrence of only Glu at residue 195. They include the following:

- the 2 protein sequencing corrections noted previously;
- the invariance on electrophoresis of β -CN (f108-209) from A¹, A², and A³ genetic variants (Groves, 1969); and
- the purification from cheese of a bitter peptide β -CN (f193-209) whose sequence is identical to the chemically corrected sequences (Gouldsworthy et al., 1996).

The previous report of Eigel et al. (1984) described 7 genetic variants. Since that revision, 3 new variants have been identified by sequence: β -CN F, previously called β -CN X (Visser et al., 1995); β -CN G (Dong and Ng-Kwai-Hang, 1998); and β -CN H (Han et al., 2000). The amino acid substitutions giving rise to all variants of β -CN are given in Table 2. In addition, Chung et

al. (1995) identified variant A⁴ in native Korean cattle using electrophoresis only; its substitutions in the A² reference protein are unknown.

Visser et al. (1995) identified β -CN F, which contains the A¹ substitution and Leu for Pro at residue 152. β -casein F was separated by preparative reverse-phase HPLC. The main differential peaks representing the 114 to 169 fragments of β -A¹ and β -X, respectively, were both purified following trypsin digestion, cyanogen bromide cleavage, and separation of the corresponding peptides representing the 145-156 sequence. The presence of Leu residue at position 152 instead of the Pro-152 in β -CN A¹ was established by fast-atom bombardment MS-MS. In accordance with internationally accepted guidelines for the nomenclature of milk proteins, the new genetic variant has been named β -CN F-5P. In a similar fashion, Dong and Ng-Kwai-Hang (1998) identified β -CN G-5P, which is similar to β -CN A¹ and F, but contains a Leu in place of Pro at either position 137 or 138, depending on the sequence assigned, as the Pro-Leu inversion is controversial. Han et al. (2000) identified β -CN H, which represents 2 substitutions relative to the corrected reference β -CN A². These are Arg25 to Cys and Leu88 to Ile. A genetic variant, discovered by Senocq et al. (2002) was also named H; so, it is proposed that the Han variant be termed H¹, and the Senocq variant be termed H². The H² variant differs from the A² variant at 2 known positions (Met93Leu and Gln72Glu) and a substitution of Gln to Glu between residues 114-169. Finally, the I variant was described by Jann et al. (2002); it contains only the Met 93Leu substitution of the H² variant.

β -Casein is the most hydrophobic of the CN. The N-terminal sequence codes for charged amino acids as well as a phosphoserine cluster. This initial sequence is different from the second half of the molecule, where neutral and hydrophobic amino acid residues abound. Calculation of the net charge at pH 6.6 indicates that the first 21 amino acids would have a net charge of about -11.5, and the C-terminal 21 amino acids (190-209) have no net charge. This molecule presents a high contrast in its sequence, one-tenth of the amino acids at the N-terminus of the protein contain one-third of the total charge, while 75% of the residues at the C-terminal one-tenth consist of hydrophobic amino acids. It is this unusual distribution of amino acids that leads to the release of β -CN from CN micelles in the cold (Aoki et al., 1990). It is important to mention that no 3-D structure from X-ray crystallography has been reported; however, a computer-generated working 3-D model has been presented (Kumosinski et al., 1993b). Perhaps the difficulty on inducing suitable crystals from this protein is due to its dependence on the envi-

ronment surrounding it and its propensity for self-association.

Addition of a glycosylation signal in the gene of β -CN has been reported (Choi and Jimenez-Flores, 1996). This modification of a bovine milk protein is an important aspect of this review because the original gene was generated from β -CN A¹ with the glycosylation signal, changing from Pro 67 to Ser 67. However, this new variant represents a man-made intervention and does not occur naturally. We suggest that nomenclature of genetic variants induced through molecular biology techniques follow the same mechanisms as those established by this Committee for naturally occurring variants, e.g., a point mutation of β -CN A¹, Pro67Ser 67.

κ -CN

The κ -CN family consists of a major carbohydrate-free component and a minimum of 6 minor components. The 6 minor components, as detected by PAGE in urea with 2-mercaptoethanol (Mackinlay and Wake, 1965; Pujolle et al., 1966; Woychik et al., 1966; Vreeman et al., 1977; Doi et al., 1979), represent varying degrees of phosphorylation and glycosylation.

κ -casein, as isolated from milk, also occurs in the form of a mixture of disulfide-bonded polymers ranging from dimer to octamers and above (Groves et al., 1992). Beeby (1964) reported the presence of free thiol groups after calcium removal by treatment with ethylenediaminetetraacetate, but other chemical analyses did not confirm this result (Swaigood et al., 1964). Sodium dodecyl sulfate-gel electrophoresis and physical measurements suggest that the native form of κ -CN is highly associated both chemically and physically (Swaigood and Brunner, 1963; Groves et al., 1992; Farrell et al., 1996) and that heat treatment of native κ -CN results in aggregation caused by free sulfhydryl-disulfide interchange (Groves et al., 1998). Reduction and S-carboxymethylation of κ -CN followed by heating can result in amyloid (fibrillar) structures (Farrell et al., 2002).

The primary structure of the reference protein of the κ -CN family is the major carbohydrate-free component of κ -CN A-1P (Figure 4); its ExPASy entry name and file number are CASK_Bovin and P02668, respectively. It consists of 169 amino acid residues as follows: Asp₄, Asn₈, Thr₁₅, Ser₁₂, Ser P₁, Pyroglu₁, Glu₁₂, Gln₁₄, Pro₂₀, Gly₂, Ala₁₄, Cys₂, Val₁₁, Met₂, Ile₁₂, Leu₈, Tyr₉, Phe₄, Lys₉, His₃, Trp₁, and Arg₅, with a formula molecular weight of 19,037. There is still some question about the presence of the N-terminal pyroglutamyl residue in the native protein, as cyclization may occur during isolation (Swaigood, 1975). In addition to protein-chemical sequencing, the cDNA of κ -CN has been sequenced (Stew-

*1	10	20
H-Glu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg-Cys-Glu-Lys-Asp-Glu-Arg-Phe-Phe-Ser-Asp-		
21	30	40
Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu-		
41	50	60
Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr-		
61	70	80
Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser-		
81	90	100
Asn-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg-His-Pro-His-		
101	110	120
Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro-		
121	130	140
Thr-Ile-Asn-Thr-Ile-Ala- <u>Ser</u> -Gly-Glu-Pro-Thr-Ser-Thr-Pro- <u>Thr</u> -Thr-Glu-Ala-Val-Glu-		
141	150	160
Ser-Thr-Val-Ala- <u>Thr</u> -Leu-Glu-Asp- <i>SeP</i> -Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-		
161	169	
Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val-OH		

Figure 4. Primary structure of *Bos* κ -CN A-1P (Mercier et al., 1973). The amino acid residues corresponding to the mutational differences in the B through J variants are given in Table 2. The arrow indicates the point of attack by chymosin (rennin). The * indicates pyroglutamate as the cyclized N-terminal. The site of post-translational phosphorylation (*SeP*) is indicated in italicized, boldface type; residues that may potentially be phosphorylated are underlined.

art et al., 1984), and the κ -CN gene sequence is complete (Alexander et al., 1988). The κ -CN signal peptide is composed of 21 amino acid residues, making the pre-form 190 amino acids in length.

The 2 common genetic variants are designated A and B (Neelin, 1964; Woychik, 1964). κ -casein B-1P differs from the A variant (Figure 4) by substitution of an Ile residue for Thr at position 136 and an Ala residue for Asp at position 148 (Mercier et al., 1973). The A variant tends to be predominant in most dairy breeds with the exception of Jersey cattle (Thompson and Farrell, 1974; Bech and Kristiansen, 1990; Ng-Kwai-Hang and Grosclaude, 2003). In alkaline gel electrophoresis, in the presence of mercaptoethanol and urea, both variants show multiple bands; the A variant possesses the greater mobility (Mackinlay et al., 1966; Swaisgood, 1975). In addition, 9 other genetic variants have been reported (Table 2). The C and E variants were characterized by digestion with cyanogenbromide (Miranda et al., 1993); C differs from A by substitution of His 97 for Arg 97; E differs from A by substitution of Gly 155 for Ser 155. The C variant has been confirmed by PCR as well (Schlee and Rottman, 1992). The letter D was ascribed to a new variant by PAGE analysis, but this variant was later found to be identical to κ -CN C (Erhardt, 1989). The incorrect identification of κ -CN D indicates the need for sequence analysis (PCR, protein, etc.) to confirm new genetic variants for all CN. κ -Casein F was discovered by PCR analysis of both Zebu and Black and White hybrid cattle (Sulimova et al., 1992). This analysis revealed 2 nucleotide changes be-

tween κ -CN A and κ -CN F: a G for T in the second position coding for Thr 145 (which yields no change in the protein) and a T for G in the second position of Asp 148 (which yields Val 148 in the F variant). This latter protein should be termed F¹, as Prinzenberg et al. (1996) by PCR analysis described a second F variant that contains the A-B changes (residues 136, 148) as well as an A for G substitution, which yields a change from Arg 10 to His 10. This variant (Arg10His) could be considered to be F², as the same researchers (Prinzenberg et al., 1996) named their next discovered variant G. This new variant (κ -CN G) was reported in Alpine breeds solely on the basis of isoelectric focusing gels (Erhardt, 1996) but was confirmed as a point mutation by PCR; this G variant causes Arg 97 of κ -CN B to be changed to Cys 97. Again, this variant could be termed G¹, as Sulminova et al. (1996) found another variant of κ -CN in yak (*Bos grunniens*) that they also termed κ -CN G; it differs from the κ -CN A by an Asp148Ala mutation, and the codons for residues 167 and 168 are different but yield no changed protein phenotype. This latter variant (Asp148Ala) could be termed G². The reasoning for the superscript nomenclature is that Prinzenberg et al. (1999) identified yet another variant in Prinzegeuer cattle and termed it κ -CN H. The H protein deviates from the A variant by a Thr135Ile mutation. Coincidentally, it is identical with κ -CN A-Zebu found by Grosclaude et al. (1974). In another study, Prinzenberg et al. (1999) described κ -CN I, which differs from the A variant by a Ser104Ala change. Finally, κ -CN J was discovered in *Bos taurus* cattle on

the Ivory Coast by Mahé et al. (1999); this variant appears to have arisen from the κ -CN B variant by a Ser155Arg mutation.

The bond sensitive to chymosin (EC 3.4.23.4) (rennin) hydrolysis occurs between Phe 105 and Met 106 (Figure 4) (Delfour et al., 1965; Jollés et al., 1968). The hydrolytic products are para- κ -CN (residues 1-105) and the macropeptide (residues 106-169). Doi et al. (1979) and Vreeman et al. (1977, 1986) have observed para- κ -CN in purified preparations of κ -CN. This is undoubtedly due to a chymosin-like proteolysis subsequent to translation, but more work must be done before concluding that para- κ -CN is a natural constituent of milk or a product of storage or of the preparatory processes. It is interesting to note that of the 11 known variants, 8 occur in the distal portion of the macropeptide, relatively far removed from the point of chymosin attack. These mutations range from positions 136 to 155 and occur in the extended portion of the molecule, which serves as a physical deterrent to coagulation prior to the action of chymosin. Small, relatively neutral changes in this portion of the molecule may not adversely affect cheese making. Perhaps more interesting are the changes in the C, F², and G¹ variants, which occur in the para- κ -CN portion of the molecule. The F² variant is functionally identical in that the net charge remains constant (Arg10His). The C variant may be of interest as Arg 97 has been implicated in the possible attraction of chymosin to the CN micelle, but the positive charge is conserved by the His 97 substitution. In a similar way, the G¹ variant in which Arg 97 is converted to Cys 97, could further influence micelle structure as the new sulfhydryl residue could promote unusual disulfide linkages close to the chymosin cleavage site.

The major component (~50%) of all κ -CN variants is generally believed to be the carbohydrate-free component. However, the post-translational modifications of κ -CN, which result in the formation of minor components, have been studied in considerable detail, and their degree of complexity is correlated with the degree of sophistication of the instrumentation used to study them. Generally, the minor κ -CN components are multiglycosylated and/or multiphosphorylated forms of the major κ -CN. Vreeman et al. (1977, 1986) concluded from their investigations that, in order of elution from DEAE-cellulose, the adsorbed κ -CN were the major components free of carbohydrate with one phosphate group followed by 6 minor components differing in degrees of glycosylation and phosphorylation. Doi et al. (1979) concluded from their fractionation of κ -CN on DEAE-cellulose that there were 4 major and 2 minor components, all containing one phosphate group and various degrees of glycosylation. The major fraction was the carbohydrate-free component.

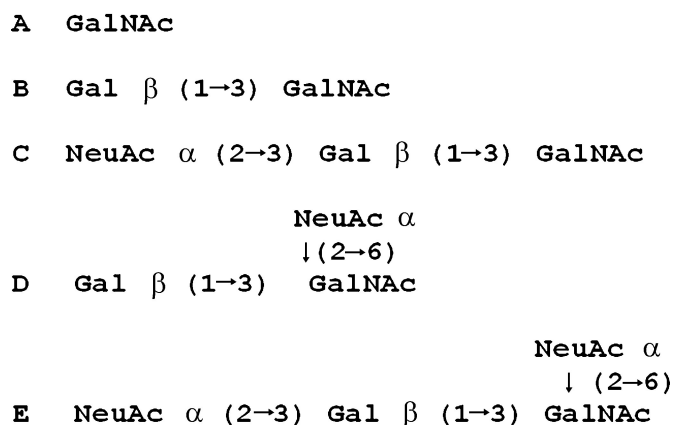


Figure 5. Reported structures for glycosyl residues attached to κ -CN macropeptide (Saito and Itoh, 1992).

Several researchers have investigated the structure of carbohydrate moieties (Tran and Baker, 1970; Fiat et al., 1972; Jollés et al., 1972, 1973; Fournet et al., 1975; Jollés et al., 1978). Fournet et al. (1975) isolated 3 oligosaccharides from κ -CN and determined the structures for 2. Saito and Itoh (1992) confirmed their structures and added 3 more; all of these structures are given in Figure 5. A composite summary of the reported glycosyl moieties, their molecular weights, and relative percentage occurrence is given in Table 3. From these data, it appears as though the complex structures C, D, and E are most prevalent. Mollé and Léonil (1995) used reverse-phase HPLC in conjunction with on-line electrospray ionization MS (EIMS) to characterize the distribution of glycosyl residues further within the κ -CN macropeptide for the A variant. They found at least 14 glycosylated forms, a glycosylated and non-phosphorylated form, and multiphosphorylated forms (1P at 78%, 2P at 20%, and 3P at 2%) totaling 18 reported species attributable to post-translational modification. The EIMS data on HPLC followed the absorbance data and confirmed the structures of Saito and Itoh (1992) except for A, the monosaccharide.

The points of attachment of the glycosyl chains have also received a good deal of attention. Again because of the high degree of heterogeneity and small amounts of some forms, the specific sites of attachment of various chains shown in Figure 5 are not clearly defined. Further advances in analytical tools, such as EIMS, could confirm this. The Thr residues at positions 131, 133, or 135 (Fiat et al., 1972; Jollés et al., 1973; Fournet et al., 1975; Kanamori et al., 1980) were initially identified as points of attachment of oligosaccharide chains through O-glycosidic linkages. If an oligosaccharide is attached to κ -CN A-1P at Thr 136, the B variant could not contain an oligosaccharide at this position, as Ile

Table 3. Summary of properties and occurrence of *O*-glycosyl residues attached to κ -CN macropeptides.

Structures of Figure 5	Reported relative percentage	Calculated Mr	Reference
A	0.8	221.2	Saito and Itoh (1992)
B	6.3	383.3	Saito and Itoh (1992); Mollé and Léonil (1995)
C	18.4	674.6	Tran and Baker (1970), Fiat et al. (1972), Fournet et al. (1979), Saito and Itoh (1992), Mollé and Léonil (1995)
D	18.5	674.6	Saito and Itoh (1992), Mollé and Léonil (1995)
E	56	965.6	Fournet et al. (1979), Saito and Itoh (1992), Mollé and Léonil (1995)

replaces Thr (Figure 5, Table 4). Although reports of monosaccharide attachments to para- κ -CN exist (Wheelock et al., 1969, 1973), para- κ -CN has generally been reported to be devoid of carbohydrate, as monoglycosylated forms of proteins can be formed artifactually (Pisano et al., 1994; Mollé and Léonil, 1995).

The carbohydrate present on colostrum κ -CN is more complex and variable than that of normal milk and was reviewed in the last report of this Committee (Eigel et al., 1984). However, a salient feature is that only Thr residues 131, 133, and 135 have been identified as points of attachment for the complex oligosaccharides bound to colostrum κ -CN (Doi et al., 1980). The earliest reported sites, Thr 131 and 133 (Pujolle et al., 1966; Mercier et al., 1973), have been confirmed, and others have been added to the list. The most complete study was conducted by Pisano et al. (1994) who demonstrated up to 6 sites for *O*-glycosylation. A summary of the reported sites is given in Table 4.

From all of these data, it would appear that for κ -CN glycosylation, structures C, D, and E are statistically most prevalent, and Thr residues 131 and 133 are the sites most populated by these glycosyl moieties. Two reasons postulated for the prevalence of glycosyl residues at the 131 and 133 sites are that the proline turns, which bound this region, maintain its surface orientation (Kumosinski et al., 1993a) and that glycosylation at other sites is restricted by the neighboring

Ile (or Val) residues (Pisano et al., 1994), which are highly prevalent in the κ -CN macropeptide. In studies with whole CN, it is important to remember that the major κ -CN bands for the A and B variants (40 to 60%) are not glycosylated; therefore, differences in the minor bands could be related to a variety of factors specific to the milk sample, not the least of which is genetic variation (Pisano et al., 1994; Mollé and Léonil, 1995).

Because of the high degree of heterogeneity and the limited amounts of the minor components of κ -CN, we feel that the precise nomenclature of these components still cannot be achieved at this time. We suggest that they be identified according to the genetic variant of the major nonglycosylated component and that isolated fractions, which contain post-translational modifications, be numbered consecutively according to either their increasing relative electrophoretic mobility in alkaline urea gels or their elution from anion exchange media in the presence of mercaptoethanol. For example, starting with κ -CN A, the nonglycosylated 1P form would be designated κ -1, and then subsequent bands would be termed κ -2, κ -3, κ -4, etc. This is in accord with most current working definitions of isolated fractions.

THE WHEY PROTEINS

The term whey proteins has been used to describe the group of milk proteins that remain soluble in milk

Table 4. Summary of the reported sites of *O*-glycosylation of κ -CN macropeptide.

Reported site	Average degree of glycosylation ¹	References
Thr 121	≤10%	Pisano et al. (1994)
Thr 131	40 to 100%	Jollés et al. (1972, 1973), Fournet et al. (1975), Kanamori et al. (1980), Zevaco and Ribadeau-Dumas (1984), Pisano et al. (1994)
Thr 133	10 to 80%	Jollés et al. (1972), Fournet et al. (1979), Kanamori et al. (1980), Zevaco and Ribadeau-Dumas (1984), Pisano et al. (1994)
Thr 135	Not found	Kanamori et al. (1980), Zevaco and Ribadeau-Dumas (1984)
Thr 136	≤10%	Kanamori et al. (1980), Pisano et al. (1994)
Thr 142	50 to 100%	Zevaco and Ribadeau-Dumas (1984), Pisano et al. (1994)
Thr 165	0 or 100%	Pisano et al. (1994)
Ser 141	Not found	Zevaco and Ribadeau-Dumas (1984)

¹Percentage residency calculated by Pisano et al. (1994).

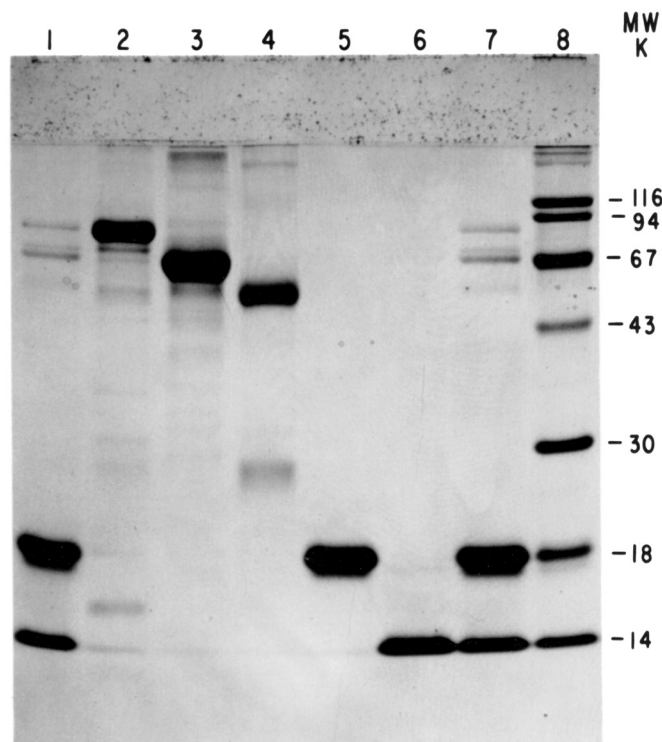


Figure 6. Discontinuous SDS-PAGE of dialyzed whey (pH 4.6) and individual purified whey proteins. 1 = dialyzed whey (pH 4.6); 2 = lactoferrin; 3 = BSA; 4 = bovine IgG, Cohn fraction II heavy chain (top) and light chain (bottom); 5 = β -LG; 6 = α -LA; 7 = dialyzed whey (pH 4.6); and 8 = standard purified proteins. Refer to Figure 10a for more details on Ig proteins. All samples were reduced with 2-mercaptoethanol (Basch et al., 1985).

serum or whey after precipitation of CN at pH 4.6 and 20°C. Traditionally, β -LG, α -LA, serum albumin (SA), Ig, and proteose-peptone fractions have been considered the major characterized components of this fraction. Because the current and most frequently used method of assessing the integrity of this fraction is SDS-PAGE, LF, a major component that is readily visualized by this technique, should be added to this list (Figure 6). It is also recognized that proteolytic fragments of CN (Eigel et al., 1984) and fat globule membrane proteins (Mather et al., 2000) occur in the traditional whey fraction, raising questions concerning the utility of this term. However, based on current knowledge, the term whey protein should be used only in a general sense to describe milk proteins soluble at pH 4.6 and 20°C. Commercial products termed whey protein isolates or concentrates are obtained from cheese manufacture at higher pH and will contain intact caseins as well as their proteolytic products, such as macropeptides and proteose-peptone fraction. Individual families, such as β -LG, α -LA, SA, and LF, should be classified according to homology with the primary sequence of their amino acid chains. Polyacrylamide or starch gel electrophoresis still can be used to characterize and identify individ-

ual members of each family. Immunoglobulins, proteins not unique to milk, are the products of B-lymphocytes and are the result of somatic gene segment rearrangement and somatic mutation. With 1 million variants, Ig lend themselves poorly to traditional biochemical characterization. Immunochemical criteria continue to be used for laboratory diagnosis and quantitation of Ig, but molecular genetics is heavily used for structural analyses.

β -LG

β -Lactoglobulin is the major protein in whey. Both the A and B genetic variants occur at high frequency in most breeds of cow, and the presence of one or the other of these 2 variants affects the properties of the milk markedly (Jakob and Puhon, 1992; Hill et al., 1996), partly because of the different physico-chemical characteristics of the β -LG molecules themselves and partly because the A variant is expressed at a higher level than the B variant (Aschaffenburg and Drewry, 1957) or the C variant (Ng-Kwai-Hang and Grosclaude, 1992; 2003; Hill et al., 1996). The latter effect suggests that there may be some differences among the non-

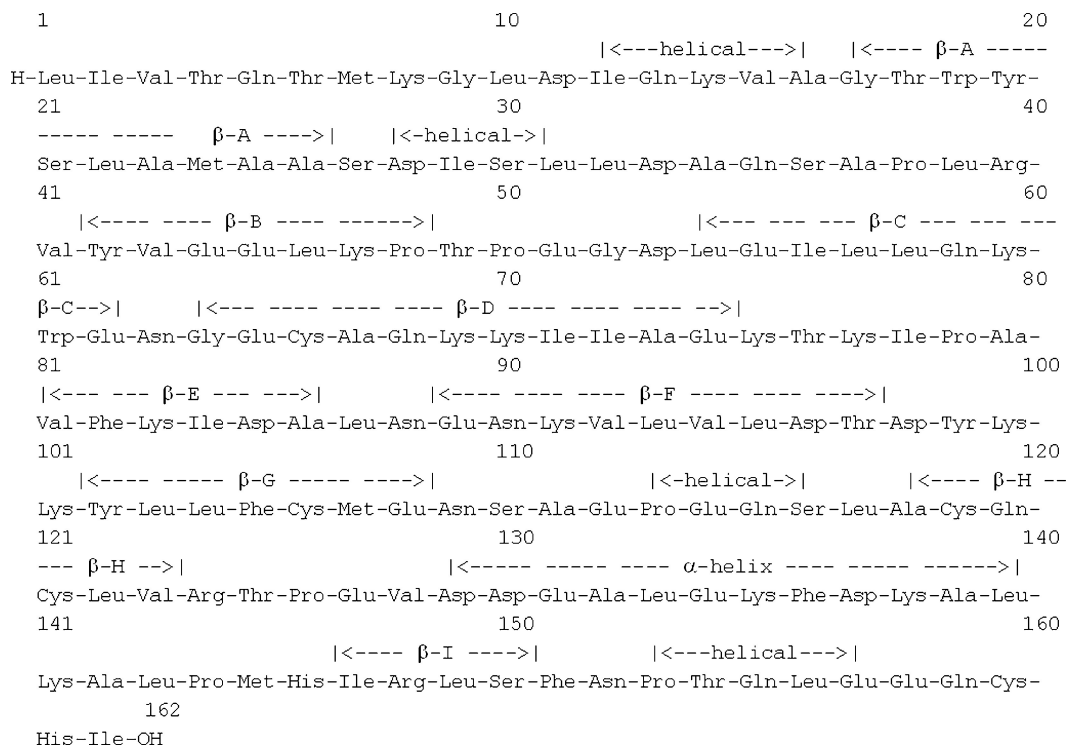


Figure 7. Primary structure of *bos* β -LG B (Eigel et al., 1984). The free sulfhydryl group is on Cys121 in the native form of the protein (Bewley et al., 1997; Brittan et al., 1997; Brownlow et al., 1997; Qin et al., 1998a,b, 1999). The two disulfide bridges are residues 66 to 160 and 106 to 120. The genetic variant substitutions are given in Table 2 for the A (Eigel et al., 1984), C (Eigel et al., 1984), D (Eigel et al., 1984), Dr. (Eigel et al., 1984), E (Eigel et al., 1984), F (Eigel et al., 1984), G (Eigel et al., 1984), I (Godovac-Zimmermann et al., 1996), J (Godovac-Zimmermann et al., 1996), and W (Godovac-Zimmermann et al., 1990) variants. The sequence positions of the major secondary structural features, α -helix, helical regions, and β -strands (β -A to β -I), are shown above the main sequence. The elements shown were determined for the triclinic (lattice X) form (Brownlow et al., 1997), and there are slight differences, particularly at the ends of the assigned structural elements, for the other crystal forms (Bewley et al., 1997; Qin et al., 1999) and at different pH (Qin et al., 1998a). The PDB (protein structural database) file for this protein is 1B8E.

coding DNA sequences of β -LG variants. The gene sequence was published by Alexander et al. (1993), and the 5' regions were explored more recently (Wagner et al., 1994; Geldermann et al., 1996). The β -LG signal peptide is composed of 16 amino acids, making the pre-form of β -LG 178 amino acids in length.

The reference protein for this family, β -LG B, consists of 162 amino acids and has the following composition: Asp₁₀, Asn₅, Thr₈, Ser₇, Glu₁₆, Gln₉, Pro₈, Gly₄, Ala₁₅, Cys₅, Val₉, Met₄, Ile₁₀, Leu₂₂, Tyr₄, Phe₄, Lys₁₅, His₂, Trp₂, and Arg₃. The calculated formula molecular weight is 18,277, and the measured molecular weight is $18,278.35 \pm 2.2$ Da (Léonil et al., 1995) or $18,277.0 \pm 0.9$ (Burr et al., 1997); its ExPASy entry name and file number are LACB_Bovin and P02754, respectively. The primary sequence shown in Figure 7 is unchanged since the 1984 review (Eigel et al., 1984). However, the disulfide bonds in the native protein are now unambiguously determined as Cys 66 to Cys 160 and Cys 106 to Cys 119, with Cys 121 as the source of the free thiol (Papiz et al., 1986; Bewley et al., 1997; Brittan et al., 1997;

Brownlow et al., 1997; Qin et al., 1998a,b, 1999). The calculated formula weight of 18,277 takes these disulfide linkages into account.

A number of new variants have been identified, and many have been sequenced (as indicated in Table 2). New variants are H (Conti et al., 1988; Davoli et al., 1988), I (Godovac-Zimmermann et al., 1996), J (Godovac-Zimmermann et al., 1996), and W (Godovac-Zimmermann et al., 1990). It should be noted that the sequences of β -LG E, F, G, and Dr do not appear to have been examined since the last review (Eigel et al., 1984), and, thus, the precise sequence site of some of the variation has not been clarified. The amino acid differences for β -LG H have not been completely verified, so the validity of its uniqueness has not been demonstrated; Ng-Kwai-Hang and Grosclaude (2003) have suggested Lys-70Asn. β -Lactoglobulin J was previously denoted as β -LG X by Baranyi et al. (1993). Another new variant is β -LG W, which has been named out of alphabetic sequence, is a "silent" variant, involving the substitution of an uncharged Ile56 residue by another un-

charged Leu56 residue (Godovac-Zimmerman et al., 1990). It was observed using the slight difference in mobility by isoelectric focusing, although no difference in mobility was observed by normal alkaline PAGE. Mass spectrometry, especially MS-MS, is probably the method of choice to confirm new variants, but it may not be suitable for β -LG W (to identify the proposed Ile/Leu substitution).

The publication of the first high-resolution X-ray crystal structures of the triclinic form (lattice X) of β -LG A/B (Brownlow et al., 1997), the orthorhombic form (lattice Y) of β -LG A, B, and C (Bewley et al., 1997), and the trigonal form (lattice Z) of β -LG A and B (Qin et al., 1998a) has verified the earlier medium resolution structure (Papiz et al., 1986) in general terms and has corrected some earlier errors. The amino acid sequences corresponding to the α -helix, the β -sheet strands (A to I), and several 3_{10} turns for the lattice X form are indicated in Figure 7. There are slight differences among the crystal lattice forms as to the details of the secondary structural elements. In all cases, the β -I strand is an important feature of the interface between the 2 monomers that constitute the dimer in all of the high resolution crystal structures. The NMR structures (Kuwata et al., 1999; Uhrínová et al. 2000) at about pH 2.5 contain the same strands and helix, confirming the polypeptide fold.

Another feature of bovine β -LG is the ability to bind hydrophobic and amphiphilic molecules ranging from hexane to palmitic acid to vitamin D (Hambling et al., 1992; Pérez and Calvo, 1995; Narayan and Berliner, 1997; Sawyer, 2003). Considerable attention has been paid to the binding of retinol (vitamin A), which is essential for mammalian growth and well being, to β -LG, and β -LG is considered a member of the lipocalin family of proteins (Sawyer, 2003). Although some retinoids and fatty acids can bind in the deep hydrophobic pocket of β -LG (Cho et al., 1994; Qin et al., 1998b; Wang et al., 1999; Kontopidis et al., 2002), there is some doubt about the biological role of this protein. The original biological role could have been related to maternal physiology, but this may have shifted to a more nutritional role for some species (Kontopidis et al., 2002).

The observation that β -LG may be glycosylated (Léonil et al., 1997) prior to milking, but probably external to the mammary epithelium, is interesting and suggests a chemical rather than a biochemical reaction. More extensive modification (lactosylation) occurs in heated milk or whey (Burr et al., 1996; Léonil et al., 1997; Morgan et al., 1998), where Lys47 and Lys91 are the most reactive, and Lys8 and Lys141 are the least reactive (Morgan et al., 1998).

The practice of naming a new variant "X" until such time as the sequence has been demonstrated should

save embarrassment and/or duplication of protein names. However, there is the possibility that such a β -LG X could be confused with the X, Y, and Z lattice forms, a nomenclature used by the crystallographers. The various engineered proteins often have slight differences at the N-terminus of the protein because of the method of synthesis. (For example, Kim et al. [1997] expressed a β -LG in which the N-terminal sequence was Glu-Ala-Glu-Ala-Tyr-Val-Thr-, whereas it is Leu-Ile-Val-Thr- in the natural proteins [Figure 7]). It is recommended that such proteins be clearly labelled so that they are not confused with the naturally synthesized proteins, because the difference in structure would give the proteins different properties, such as electrophoretic mobility.

α -LA

The whey protein α -LA has a specific and defined physiological function in the mammary gland. Within the Golgi apparatus of the mammary epithelial cell, α -LA interacts with the ubiquitously expressed enzyme β -1,4-galactosyltransferase to form the lactose synthase complex. α -lactalbumin modifies the substrate specificity of β -1,4-galactosyltransferase, allowing the formation of lactose from glucose and UDP-galactose. The constitutive function of β -1,4-galactosyltransferase, to glycosylate glycoproteins and glycolipids is reversibly altered by combining with α -LA in a 1:1 molar ratio. The production of lactose, its function as the major osmolyte of milk, and the function of the lactose synthase complex, have been the topics of a number of reviews (Brew and Hill, 1975; Hill and Brew, 1975; Jones, 1977; Kuhn et al., 1980).

Bovine milk contains α -LA at a concentration of approximately 1.2 to 1.5 g/L (Jenness, 1974). The protein has been sequenced, and the nucleotide sequence has been confirmed (Vilotte et al., 1987; Brew et al., 1970; Bleck and Bremel, 1993a). The mature α -LA (Figure 8) is a 123-amino acid globular protein (Brew et al., 1970). The α -LA signal peptide is composed of 19 amino acids, making the pre-form of α -LA 142 amino acids in length (Gaye et al., 1987; Hurley and Schuler, 1987). The mature α -LA protein has 2 predominant genetic variants (A and B) that have been confirmed by sequence analysis (Table 2) (Bhattacharya et al., 1963). The B variant is present in the milk of most *Bos taurus* cattle, and both the A and B variants are found in *Bos indicus* cattle (Jenness, 1974). α -lactalbumin A variant is present at a low frequency in some Italian and Eastern European *Bos taurus* breeds (Mariani and Russo, 1977). The A variant contains a Glu at position 10 of the mature protein, and the B variant has an Arg substitution at that position (Gordon, 1971). A third genetic variant,

1	10	20
H-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Arg-Glu-Leu-Lys-Asp-Leu-Lys-Gly-Tyr-Gly-Gly-		
21	30	40
Val-Ser-Leu-Pro-Glu-Trp-Val-Cys-Thr-Thr-Phe-His-Thr-Ser-Gly-Tyr-Asp-Thr-Gln-Ala-		
41	50	60
Ile-Val-Gln-Asn-Asn-Asp-Ser-Thr-Glu-Tyr-Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Ile-Trp-		
61	70	80
Cys-Lys-Asp-Asp-Gln-Asn-Pro-His-Ser-Ser-Asn-Ile-Cys-Asn-Ile-Ser-Cys-Asp-Lys-Phe-		
81	90	100
Leu-Asp-Asp-Asp-Leu-Thr-Asp-Asp-Ile-Met-Cys-Val-Lys-Lys-Ile-Leu-Asp-Lys-Val-Gly-		
101	110	120
Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys-Ala-Leu-Cys-Ser-Glu-Lys-Leu-Asp-Gln-Trp-Leu-Cys-		
123		
Glu-Lys-Leu-OH		

Figure 8. Primary sequence of *Bos* α -LA B (Brew et al., 1970; Vanaman et al., 1970). The disulfide bridges in the molecule are between positions 6 and 120, 28 and 111, 61 and 77, and 73 and 91. The amino acid residues corresponding to the differences in genetic variants A and C are given in Table 2. The PDB (protein structural database) file for this protein is 1F6S.

α -LA C, has also been reported but not confirmed by DNA or protein sequencing (Bell et al., 1981). This variant was identified in Bali cattle (*Bos javanicus*). The C variant was reported to differ from the B variant by having either an Asn for Asp or a Gln for Glu substitution. The B variant is the reference protein for the family and is composed of the following amino acid residues: Ala₃, Arg₁, Asn₈, Asp₁₃, Cys₈, Gln₆, Glu₇, Gly₆, His₃, Ile₈, Leu₁₃, Lys₁₂, Met₁, Phe₄, Pro₂, Ser₇, Thr₇, Trp₄, Tyr₄, Val₆ (Brew et al., 1970). Both the A and B variant contain 4 disulfide bonds. The B variant has a formula molecular weight of 14,178; its sequence is given in Figure 8 and has been corrected since the last report to take into account the nucleic acid sequences noted previously. The ExPASy entry name for α -LA is LACA_Bovin, and its file number is P00741. α -lactalbumin has a very high content of the essential amino acids (Trp, Phe, Tyr, Leu, Ile, Thr, Met, Cys, Lys, and Val). Essential amino acids account for 63.2% of the total amino acid content compared with just 51.4% for total CN (Heine et al., 1991). The amino acid composition of bovine α -LA and its 72% sequence identity to human α -LA makes it an ideal protein for the nutrition of human infants (Heine et al., 1991).

A small percentage of the α -LA found in the milk of cattle is glycosylated on an Asn residue (Barman, 1970). The N-linked glycosylation signal occurs at amino acids 45 to 47 (Asn-Gln-Ser) of mature α -LA (Figure 8). The reason why only a small portion of the protein is glycosylated is not clear. The mechanism that has been suggested is that the glycosylation machinery has poor accessibility to the glycosylation site in the mature folded protein (Pless and Lennarz, 1977). However, examination of its x-ray structures (Chrysina et al., 2000) does not support this concept. The potential physiological relevance of the glycosylation has not been deter-

mined. Bovine α -LA is not phosphorylated in its native form (Bingham et al., 1988). However, α -LA becomes a good substrate for CN kinase in vitro after it has been reduced and carboxymethylated (Bingham et al., 1988).

In dairy cattle, the concentration of α -LA in milk decreases near the end of a lactation (Caffin et al., 1985; Regester and Smithers, 1991). This is opposite of what occurs for the other major bovine milk proteins; their concentrations tend to increase as a lactation progresses (Davies and Law, 1980). The decline in α -LA concentration is correlated with the decline observed in the concentration of milk lactose at the end of a lactation. Lower concentrations of α -LA have also been observed in cows that have mammary infections (Caffin et al., 1985).

The protein structure, amino acid sequence, and DNA sequence of α -LA are very similar to that of the c-type lysozymes (McKenzie and White, 1991), and the predicted 3-D structure of each of these 2 proteins has been analyzed in great detail (Acharya et al., 1989; McKenzie and White, 1991). Bovine α -LA and bovine lysozyme show a 62.6% similarity and 35.8% identity at the amino acid level.

α -Lactalbumin has been identified as a calcium metalloprotein (Hiraoka et al., 1980). The binding of calcium, zinc, and other metals to α -LA and calcium's function in the formation of native α -LA have previously been reviewed (Brew and Grobler, 1992; Permyakov and Berliner, 2000). There is evidence that the binding of calcium is necessary for proper folding and disulfide bond formation of the native protein (Chrysina et al., 2000). A 2.2-Å resolution crystal structure for bovine α -LA has been elucidated (Pike et al., 1996; Chrysina et al., 2000). The folding pattern for bovine α -LA is distinct from the X-ray structures that were determined for baboon, human, guinea pig, and goat α -LA (Acharya

et al., 1989, 1990, 1991; Harata and Muraki, 1992; Pike et al., 1996; Farrell et al., 2002). This analysis has identified a distinct calcium-binding site within the native form of α -LA. There is good evidence that α -LA may also be a zinc-binding protein (Ren et al., 1993). In addition to its native 3-D structure, α -LA can form a stable conformational state termed a molten globule under slightly denaturing conditions (Kuwajima, 1989, 1996). This state is a conformation of α -LA that occurs as an intermediate step during the formation of native protein and has been studied and reviewed in great detail as a model for the process of protein folding (Kuwajima, 1989, 1996; Wijesinha-Bettoni et al., 2001; Farrell et al., 2002).

The crystal structure for lactose synthase has also been elucidated (Ramakrishnan and Qasba, 2001). The structure was created using recombinant mouse α -LA and the catalytic domain of recombinant bovine β -1,4 galactosyltransferase. The analysis indicated that the major role of α -LA in the lactose synthase complex is to hold the glucose molecule and inhibit the binding of N-acetyl-glucosamine to β -1,4 galactosyltransferase, thus allowing the attachment of glucose to UDP-galactose to form lactose.

Site-directed mutagenesis and the expression of mutant α -LA in bacteria has allowed for detailed analysis of important functional α -LA amino acids. Mutations made to Glu 117 or Trp 118 reduced the binding affinity of α -LA for β -1,4-galactosyltransferase, while mutations to Phe 31 or His 32 have effects on the ability of α -LA to promote glucose binding to the lactose synthase complex (Grobler et al., 1994).

The genes encoding α -LA and lysozyme are found on bovine chromosome 5 (Threadgill and Womack, 1990). The α -LA gene is composed of 4 exons that are transcribed into a 724 base mRNA (Gaye et al., 1987; Hurley and Schuler, 1987; Vilotte et al., 1987). The coding region of α -LA along with approximately 2.0 kb of 5' flanking DNA and 350 bp of 3' flanking region have been sequenced (Vilotte et al., 1987; Bleck and Bremel, 1993b). Two DNA variations have been identified in the region of the gene encoding the bovine α -LA mRNA. In addition to the DNA variation that produces the A and B variants of α -LA (Osta et al., 1995a), a second DNA variation has been identified in the section of the gene that encodes the 5' untranslated region of the mRNA. This polymorphism occurs at position +15 from the transcription start site (Bleck and Bremel, 1993a,b).

The importance of α -LA for the normal function of the mammary gland and normal secretion of milk has been shown using mice that have the gene encoding α -LA "knocked out" (Stinnakre et al., 1994; Stacey et al., 1995). Homozygous mice containing no functional copies of the α -LA gene produce a milk that contains

no lactose, is extremely viscous, and cannot be removed from the mammary gland by the developing pups. Homozygous mice that contain a single copy of the functional α -LA gene produce a milk that contains less lactose and has a significantly higher solids content. These studies verify the importance of α -LA not only as a protein source in milk, but also as a regulator of lactose production and the secretion of milk.

SA

Serum albumin, a major protein found in blood serum, occurs in all body tissues and secretions; it has a principal role in the transport, metabolism, and distribution of ligands (Carter and Ho, 1994); it contributes to osmotic pressure of blood (Carter and Ho, 1994); and it imparts free radical protection. Bovine SA as found in milk, is physically (Polis et al., 1950) and immunologically (Coulson and Stevens, 1950) identical to blood SA. Bovine SA represents about 1.5% of total milk protein and about 8% of total whey protein. Prior to 1990, the complete amino acid sequence of BSA was thought to contain 582 amino acids and to have a calculated molecular weight of 66,267 (Brown, 1975, 1977; Reed et al., 1980). However, based on EIMS molecular weight values, Hirayama et al. (1990) observed an error in this sequence and amended the sequence to 583 amino acid residues with 17 disulfide bonds and a corrected molecular weight of 66,399. Hirayama et al. (1990) also reported a reversed amino acid sequence for the 94th and 95th positions. The amino acid composition of the latest version is Ala₄₇, Arg₂₃, Asn₁₄, Asp₄₀, Cys₃₅, Gln₂₀, Glu₅₉, Gly₁₆, His₁₇, Ile₁₄, Leu₆₁, Lys₅₉, Met₄, Phe₂₇, Pro₂₈, Ser₂₈, Thr₃₃, Trp₂, Tyr₂₀, Val₃₆. Human SA (**HSA**) contains 583 amino acids and has, taking the known 17 disulfide bonds into account, a formula molecular weight of 66,399. The corrected amino acid sequence for BSA is given in Figure 9; its ExPASy entry name and file number are ALBU_Bovin and P02769, respectively.

The amino acid sequence of SA has also been reported for several mammalian species. Carter and Ho (1994) reported that sequence homologies were high among those mammals studied, as was the conservation of the characteristic repeating disulfide pattern. Sequence identity between BSA and ovine SA was 92.4 and 76% between HSA and BSA (from Table 2, Carter and Ho, 1994). An amino acid sequence compiled from cDNA data for equine SA revealed sequence identities between equine SA and HSA, BSA, porcine SA, ovine SA, and rat SA of 76, 74, 76, 75, and 76%, respectively (Ho et al., 1993). The BSA signal peptide is composed of 18 amino acids, making the pre-form of BSA 607 amino acids in length.

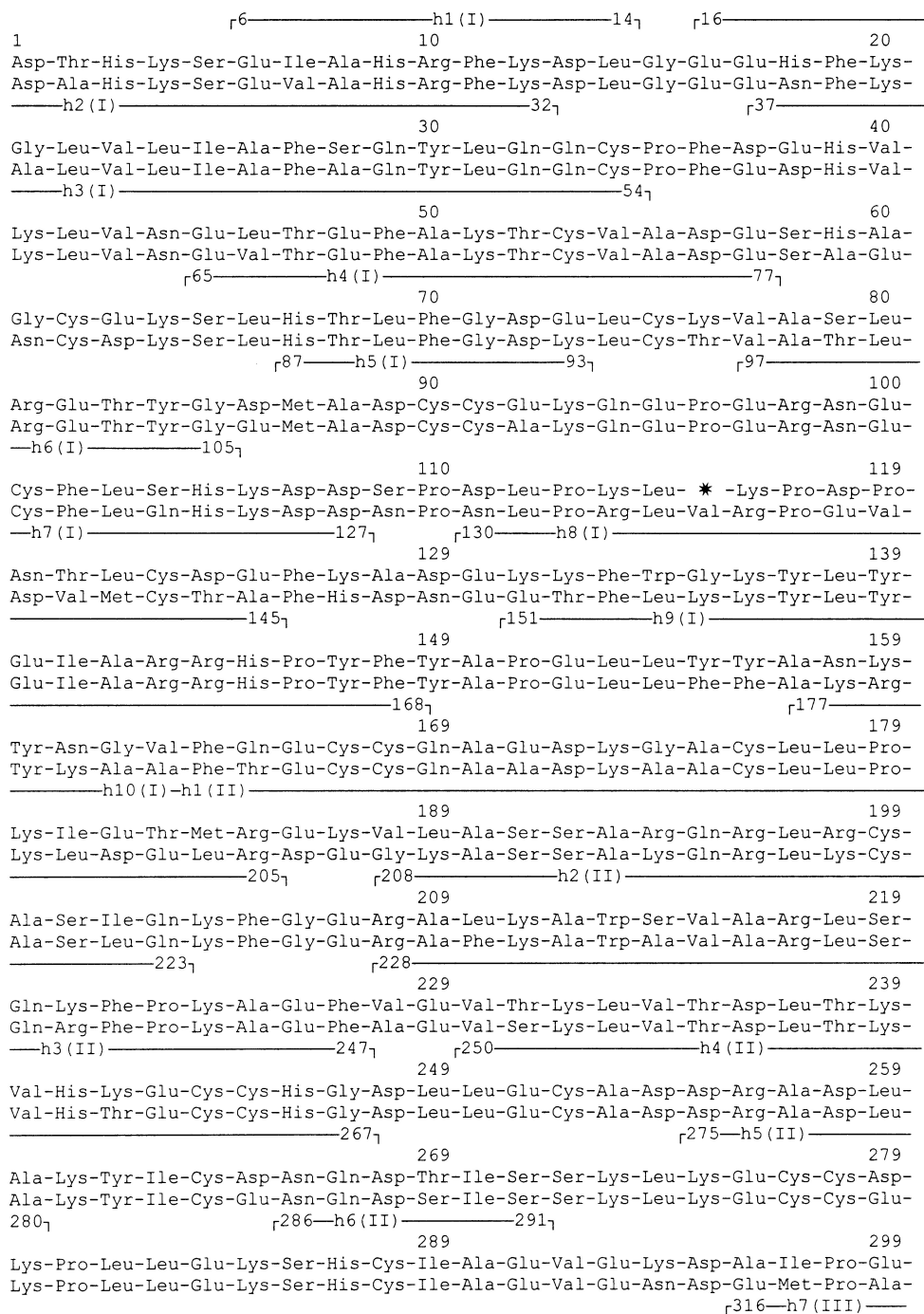


Figure 9. Normalized albumin sequence homology: bovine/human aligned sequences illustrating conserved amino acids and invariant residues. Upper line = BSA, lower line = human serum albumin. Disulfide bonds exist between Cys at aligned positions of 53 to 62, 75 to 91, 90 to 101, 123 to 168, 167 to 176, 199 to 245, 244 to 252, 264 to 278, 277 to 288, 315 to 360, 359 to 368, 391 to 437, 436 to 447, 460 to 476, 475 to 486, 513 to 558, and 557 to 566. Cys-34 is free and unbound. The PDB (protein structural database) file for human serum albumin is 1A06.

The 3-D structure of HSA has been determined by X-ray crystallography (Ho et al., 1993), and, based on sequence work, BSA could be similar. All reported SA

proteins (except lamprey) are comprised of 3 homologous domains termed I, II, and III (Carter and Ho, 1994). Carter and Ho (1994) verified that each SA do-

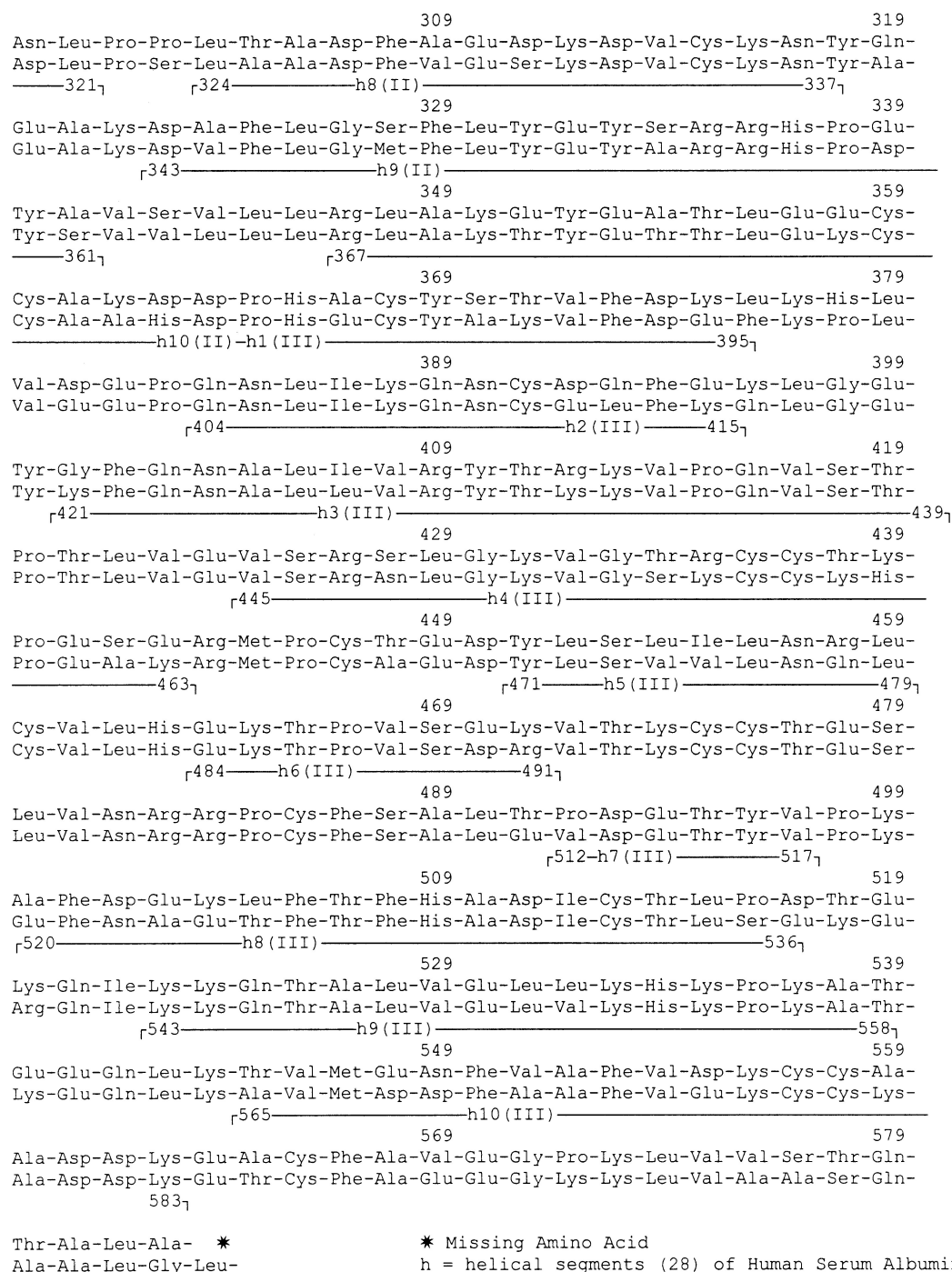


Figure 9 (Continued). Normalized albumin sequence homology: bovine/human aligned sequences illustrating conserved amino acids and invariant residues. Upper line = BSA, lower line = human serum albumin. Disulfide bonds exist between Cys at aligned positions of 53 to 62, 75 to 91, 90 to 101, 123 to 168, 167 to 176, 199 to 245, 244 to 252, 264 to 278, 277 to 288, 315 to 360, 359 to 368, 391 to 437, 436 to 447, 460 to 476, 475 to 486, 513 to 558, and 557 to 566. Cys-34 is free and unbound. The PDB (protein structural database) file for human serum albumin is 1A06.

main contained 2 subdomains (1A, 1B, etc.) for a total of 6 subdomains. Each domain contains 10 helical regions, 2 overlapping for a total of 28 helices, which are

extensively cross-linked by 17 disulfide bonds (Eigel et al., 1984). In addition, there are 9 loops (L1 to L9) that are held together by disulfide bonds (Carter and

Ho, 1994). The domains are assembled to form a heart-shaped molecule within the pH range of 4.5 to 8.0 (Carter and Ho, 1994). Human SA undergoes a major reversible conformational isomerization with changes in pH, adopting a more or less compact configuration with varying conditions (Olivieri and Craievich, 1995). Measurements of the native molecular structure are roughly that of an equilateral triangle with sides of ~80 Å (each side) and depth of ~30 Å (Carter and Ho, 1994). Carter and Ho (1994) published a depiction of the molecule that shows the 3 domains, the subdomains, and the 9 loops (L1 to L9) that should be referred to when discussing dimensional aspects of this molecule. Disulfide bonds exist between Cys at positions 53 to 62, 75 to 91, 90 to 101, 123 to 168, 167 to 176, 199 to 245, 244 to 252, 264 to 278, 277 to 289, 315 to 360, 359 to 368, 391 to 437, 436 to 447, 460 to 476, 475 to 486, 513 to 558, and 557 to 566. Cys-34 is free and unbound.

Ig

Introduction and general comments. Analysis of either whole milk or whey protein by SDS-PAGE reveals distinct bands for the Ig fraction (Figures 6 and 10a). The Ig fraction accounts for about 1% of total milk protein or about 6% of total whey protein.

The nomenclature of Ig in cow milk reported in the fourth and fifth revisions by the Committee on the Nomenclature and Methodology of Milk Proteins of the Dairy Foods Research Section (Whitney et al., 1976; Eigel et al., 1984) has been based on the proposed nomenclature for domesticated Bovidae (Aalund et al., 1971), as well as on the WHO-proposed rules for Ig of animal origin (Ambrosius et al., 1978). Sequences and comparison with those of Ig from other species may be viewed in the original references in GenBank and in compilations of sequences of proteins of immunological interest (Kabat et al., 1991). In addition, the retrieval and deposition of new information has been facilitated by the establishment of databases on the World Wide Web, such as that provided by the National Centre of Biotechnology Information (<http://www.ncbi.nih.gov>). Specifically, the Ig nomenclature of various species, including cattle, is available on the IMGT web site (see previous) and on the Comparative Immunoglobulin Workshop web site (www.medicine.uiowa.edu/CIgW). In the latter, accession numbers are given, and these are linked to the sequences in GenBank. By agreement, most nomenclature now follows guidelines proposed by Le Franc and Le Franc (2001) for human Ig and Ig genes (Table 5).

Overview of Ig Structure

Studies to date indicate that the basic structures of Ig in *Bos* are similar to those of human origin (Butler,

1969, 1974, 1983, 1985; Larson, 1992). Immunoglobulins occur as polymers or protomers of a basic “Y-shaped” unit composed of 4 polypeptide chains linked by inter- and intramolecular disulfide bonds (Figure 10b). The monomers consist of 2 identical heavy chains (**H**) with molecular weights ranging from about 55 to 76 kDa, depending on the class, and 2 identical light chains (**L**) with molecular weights of 22.5 to 27.3 kDa (Butler, 1985; Figure 10a,b). The sizes of the polypeptide chains are normally determined by SDS-PAGE, so heavily glycosylated chains are retarded. In addition, incompletely reduced chains may alter the calculated sizes. The H contains a constant (**C**) region composed of 3 to 4 domains of about 110 amino acids and a single N-terminal variable (**V**) region domain. Light chains are either λ or κ ; each are encoded at separate unlinked loci and are composed of one C-terminal C domain and one N-terminal V region domain. The V domains of H and L converge to form the antigen-binding site. Because each monomeric unit is composed of 2 H and 2 L, each monomer is bivalent.

Although the paired V domains determine the binding specificity of the Ig molecule, the C regions determine the isotype (class) of the Ig, e.g., IgM, IgG, etc., as well as the various biological activities of Ig (Butler, 1985). Allotypes are genetic variants within the population, much like erythrocyte blood groups, and allotypic determinants are usually found in the C regions of the H and L. Idiotypes are determinants associated with the re-arranged VDJ and VJ regions of Ig (see subsequently); their expression often correlates with a particular antibody specificity.

The N-terminal V region domains, shown in Figure 10b, are actually encoded by 3 (V, D, and J) gene segments for the H and 2 (V, J) gene segments for either κ or λ L. These segments are multigenic in any one individual, e.g., humans have 95 V_H segments, 30 D_H segments, and 6 J_H segments. These segments must be somatically rearranged during B-cell development to produce one productive VDJ and one VJ rearrangement per B-cell. Because these rearranged gene segments encode the V_H and V_L domains that determine binding specificity, this multigenic arrangement can account for 10^7 different antibodies. Added to this is the fact that sequences can vary at the junction of V to D and D to J in the H and V to J in the L (“junctional diversity”). Furthermore, these re-arrangements are the target of somatic hypermutation with a rate that is 10^7 higher than the eucaryotic germline mutation rate. Thus, up to 10^{12} different antibody specificities can be produced by one individual. Species differ in the degree to which these mechanisms are used in repertoire development, and in cattle, the pattern is unclear. This is briefly

Table 5. Bovine Ig isotypes.¹

Original description	Current designator	Chain and allotype	Gene	Serological allele	Sequence allele	GenBank account number	Major features and other information
IgG1	IgG1 ^a IgG1 ^b	$\gamma 1^a$ $\gamma 1^b$	IGHG1 IGHG1		G1 * 01 G1 * 02	S82409	Hinge, Arg218; Thr226 Hinge; Thr218; Pro224; Pro226
IgG2a(A1)	IgG2 ^a	$\gamma 2^a$	IGHG2	G2 ^a	G2 + 01	S82407	CH3; intradomain loop hepatappetide; Arg419
IgG2a(A2)	IgG2 ^b	$\gamma 2^b$	IGHG2	G2 ^b	G2 + 02		Middle hinge, CH3, Glu419
IgG2b/IgG3	IgG3 ^a IgG3 ^b	$\gamma 3^a$ $\gamma 3^b$	IGHG3 IGHG3		G3 * 01 G3 * 02	U63638 U63639	37 amino acid hinge 6 amino acid substitutions; 8 INV3 insertion
IgA	IgA	α	IGHA			AF109167	PstI RFLP generates polynucleotides of 6.0 and 6.3 kb suggestive of sequence alleles
IgM	IgM	μ	IGHM			U63637	
IgE	IgE	ϵ	IGHE			U63640	PstI, EcoRI and BAMI RFLP show polymorphism suggestive of sequence alleles
IgD	IgD	δ	IGHD			AF515672 and AF411240	In cattle, IgD is preceded by a switch region unlike mouse or human

¹Refer to comparative Ig workshop Web site (www.medicine.uiowa.edu/CIgW).

²The superscripts a and b relate to the nomenclature of Ig allotypes as described in text.

discussed subsequently and is discussed in detail elsewhere (Butler, 2004).

Each domain of an Ig, including the V regions, is folded in a common manner, known throughout biochemistry as the Ig fold. This folded structure is composed of a “ β -barrel” of antiparallel β -pleated sheets joined at their ends by flexible polypeptide segments. In the case of the V domains, these flexible polypeptides converge at the N-terminus of the domain to form the antigen-binding site (see previous; Janeway et al., 2001).

The junctions between domains in all Ig are more susceptible to proteolytic cleavage than the β -barrel domain structure. Thus, a wide variety of proteases attack these junctions, producing a spectrum of separate domain products. Best known are papain, trypsin, and pepsin, which attack the so-called hinge of IgG between the first and second C region domains (dark area, Figure 10b). Depending on the precise site of cleavage, a single divalent F(ab)₂ fragment plus a 30-kDa pFc' fragment is generated (typical for pepsin), whereas, in other cases, 2 separate Fab fragments and one Fc fragment is generated (typical for papain). These fragments are useful in preparing immunochemical reagents and for identifying the location of allotypic or idiotypic determinants or testing for subclass-associated biological functions. Immunoglobulin M, IgE, and IgA are more resistant to proteolytic activity, although the latter is often attached by various “IgA proteases” (Kilian and Russell, 1998).

Another feature of Ig is that the C-terminal domain of each class (isotype) can be transcribed in 2 forms. One form contains a short membrane anchor (membrane exon); the other is transcribed with an even shorter secretion exon. The former permits any particular class of Ig to be expressed as a B-cell receptor on B

lymphocyte membranes; the latter tailors Ig for secretion from the B-cell.

Interestingly, many proteins of immunological interest are encoded by genes of the Ig supergene family and include the following: a) B-cell surface receptors, b) the membrane proximal portion of the major histocompatibility complex protein, c) the T-cell receptor, d) most Fc receptors (see subsequent), e) one family of adhesion molecules, f) some cytokine receptors, and g) β_2 -microglobulin [a polypeptide chain associated with class I major histocompatibility complex molecules (Petersen et al., 1972)]. The latter was first crystallized from milk as lactollin (Groves et al., 1963) and was reviewed in the last report of this Committee (Eigel et al., 1984).

L and H Components of Bovine Ig

The extreme heterogeneity of Ig reduces the value of physical and chemical parameters for their characterization. Not surprisingly, identification of Ig has traditionally been based on immunochemical criteria rather than more conventional biochemistry. Increasingly, progress in the characterization of bovine Ig and the genes encoding them, is based on molecular genetic methods. To overcome the problem of polyclonal heterogeneity, heterohybridomas secreting bovine IgM, IgE, IgA, IgG1, and IgG2 molecules have been produced (Srikumaran, 1983, 1987; Guidry et al., 1986; Goldsby et al., 1987). Thus, homogeneous Ig proteins are now available for biochemical studies.

Although κ and λ L are nearly equally represented in humans and swine, sequence analyses by Hood et al. (1967) suggested that >90% of all bovine L are of the λ type. This was confirmed in the last decade, and molecular genetic methods were used to characterize

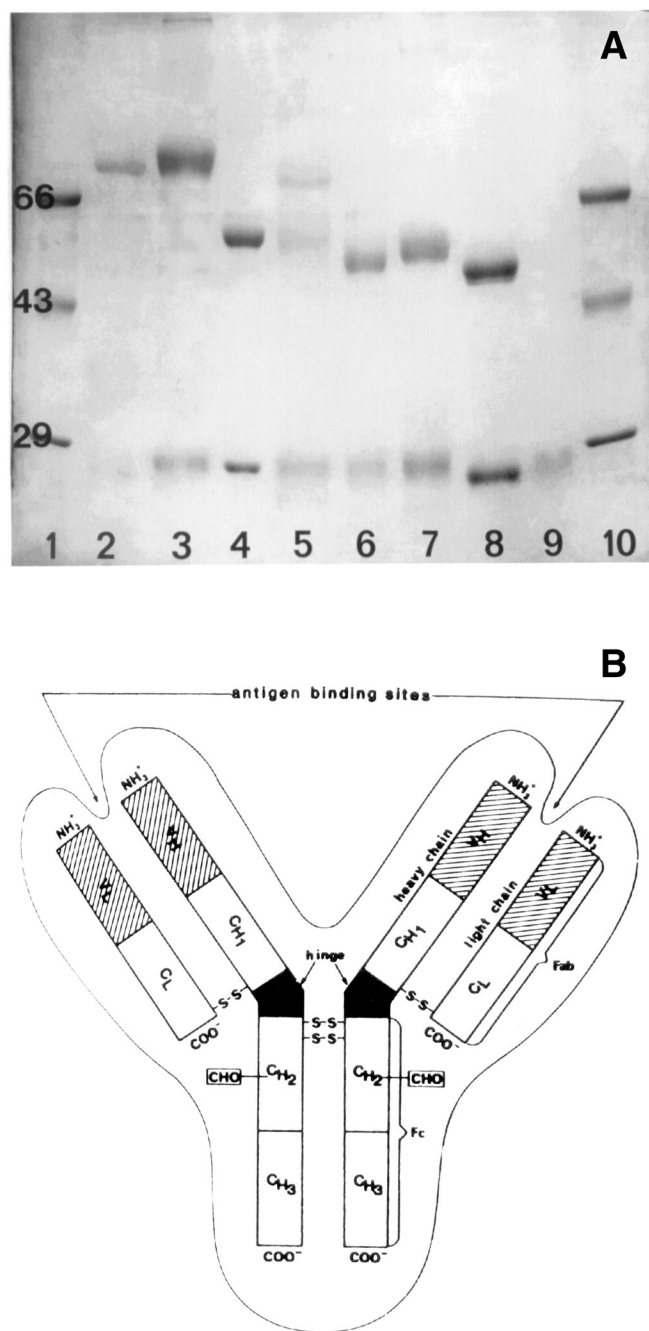


Figure 10 a) Polypeptide composition of most bovine and reference Ig revealed by SDS-PAGE. Lane 1 = reference standards with molecular size indicated in kiloDaltons, lane 2 = human monoclonal IgM, lane 3 = bovine IgM, lane 4 = human monoclonal IgA, lane 5 = bovine SIgA (largest molecular weight peptide is the secretory component), lane 6 = bovine IgG2, lane 7 = bovine IgG1, lane 8 = human IgG, lane 9 = bovine light chains, and lane 10 = reference standards. Note the smaller size of the IgG2 heavy chain. Data for IgG3, IgD, and IgE are not shown. b) Model of the basic 7S Ig molecule showing 2 heavy (H) and 2 light (L) chains joined by disulfide bonds. V = variable region; C = constant region. Subscripts 1, 2, and 3 refer to the 3 C regions of the H. CHO = carbohydrate groups. Fab refers to the (top) antigen-specific portion of the Ig molecule, and Fc refers to the cell-binding effector portion of the Ig molecule (Guidry, 1985; Larson, 1992).

many V λ gene segments. There are data on bovine λ chain sequences including those of the V regions (Ivanov et al., 1988; Sinclair et al., 1995; Parng et al., 1996). Additional information is accumulating on usage of the

V region genes encoding the V_H, and V λ repertoire in cattle. The majority of the expressed bovine Ig H V regions are encoded by the V_H4 gene family (Saini et al., 1997; Sinclair et al., 1997), which is a departure

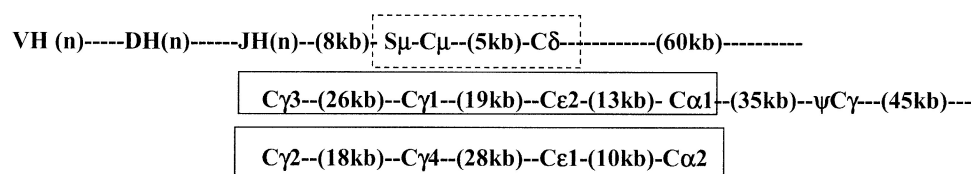
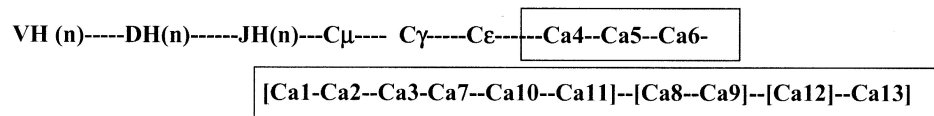
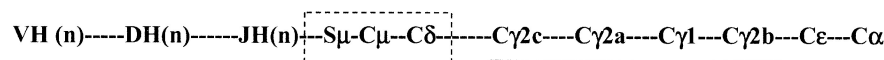
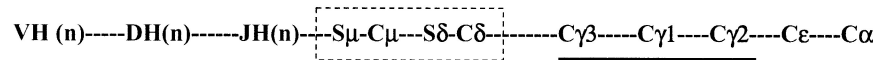
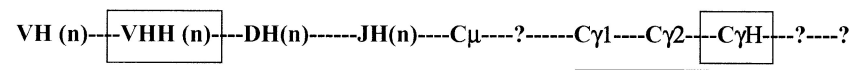
Human**Rabbit****Mouse****Bovine****Dromedary**

Figure 11. Genomic organization of the heavy chain locus in cattle compared with that of human, rabbit, mouse, and camel. Highlighted area in the human depicts the duplication in the constant region locus, while the highlighted area for rabbit depicts the duplication of genes encoding IgA; here a = α . The region enclosed by interrupted lines is the $S\mu$ - $C\delta$ region. Note that only cattle have a $S\delta$ segment (Butler, 2004).

from the usage of V_H3 in swine and rabbits. Especially noteworthy is the exceptionally long CDR3 region reported for some V regions (Saini et al., 1999). This has given rise to the idea of an additional flexible V region domain that might function in the “induced fit” concept of the antibody-binding site (Butler, 1997, 2004). Molecular genetic approaches have also allowed the genes encoding the bovine Ig C regions to be cloned, sequenced (Table 5), and their genomic organization established (Figure 11; Butler, 2004). Initial studies of this type shed light on the repertoire of genes encoding the major classes and subclasses (Knight et al., 1988; Knight and Becker, 1987), while subsequent studies provide sequence information for individual genes (Kacskovics and Butler, 1996; Brown et al., 1997; Mousavi et al., 1997; Rabbani et al., 1997; Symons et al., 1987, 1989). Zhao et al. (2002) have clarified the organization of the bovine H locus (Figure 11) and more recently characterized bovine IgD. These studies have collectively shown that only 3 IgG subclasses are functionally encoded in cattle (see Table 5 and CIgW web site).

Allotypic variants (A1/A2) associated with both IgG2 and IgG1 have also been reported by various investigators (Blakeslee et al., 1971; Rapacz and Hasler-Rapacz,

1972; Wegrzyn, 1978; Butler and Heyermann, 1986; Butler et al., 1987; Heyermann and Butler, 1987; Heyermann et al., 1992; Butler et al., 1994; Kacskovics et al., 1995; Kacskovics and Butler, 1996). Both allotypes of IgG2a (now designated IgG2^a and IgG2^b; Table 5) have a short hinge region and a truncated C_H2 domain, which is characteristic of all reported sequences of IgG2 but not IgG1 proteins in cattle (Heyermann et al., 1992). However, the C_H3 domain of IgG2b (now IgG2^b, Table 5) and IgG1 show a surprising homology in the occurrence of 5 substitutions in comparison with the same domain in the A1 allotype. Heterogeneity of bovine IgG2, together with isotype and allotype bias in anti-IgG reagents, have been shown to influence the outcome of serological tests for identification and quantification of IgG (Bokhout, 1975a, b; Bokhout and van Astenoordijk, 1979; Butler et al., 1994). The structural differences in IgG1 and the allotypes of IgG2 suggest differences in biological activity. Most striking are differences in Fc receptor specificity and transport by the acinar epithelial cells of mammary gland that may use FcRn for this function (Butler, 1997; Kacskovics et al., 2000; Mayer et al., 2002; Butler and Kehrli, in press; see subsequent section).

Although Ig consist of a basic 4-polypeptide chain unit of 2 H and 2 L, both IgM and IgA can form covalent polymers, pentamers, and dimers, respectively, with the addition of a 15-kDa polypeptide chain known as the J (joining) chain. The J chain plays an important role in assembly of polymeric Ig and their selective transport across epithelial cell layers. The J chain is disulfide-linked and covalently associated with the carboxyterminal tail of μ -chains and α -chains (Kulseth and Rogne, 1994). Cloning and characterization of bovine Ig J chain cDNA and its promoter region have been reported (Kulseth and Rogne, 1994) and show extensive homology with the J chain from human mouse, rabbit, and bullfrog, including a highly conserved propensity to form β -sheets. Earlier work also documented the evolutionary conservation of the J chain among higher vertebrates, as evidenced by cross-reaction of J chains from dog, cat, cow, goat, sheep, pig, horse, hedgehog, guinea pig, rat, mouse, and chicken by using rabbit antisera to human J chain (Kobayashi et al., 1973). Methods for isolation of pentameric IgM as well as its J-chain from bovine milk or colostrum have been reported (Mukkur and Froese, 1971; Komar and Mukkur, 1975; Kanno et al., 1976). The secretory component (SC) is found in milk both free and in association with IgA (Eigel et al., 1984); it is known to be a part of a polymeric Ig receptor (Kulseth et al., 1995). Its ExPasy file name is P81265.

Ig in Bovine Milk and Colostrum

Of the 5 isotypes of immunoglobulins in mammals, all have now been identified in cattle (see CIGW web site); IgG, IgA, and IgM have been characterized in milk, and their concentrations have been described and reviewed (Table 6). Changes in the level and relative proportions of the Ig in colostrum compared with milk during lactation or resulting from immunization or infections have been studied (Mackenzie and Lascelles, 1968; Butler et al., 1972; Guidry et al., 1980; Hidirolglou et al., 1992). This topic and the origin of both Ig and immunocytes in milk, especially in cattle, have been reviewed in detail (Butler, 1998; Butler and Kehrli, in press).

Immunoglobulin transfer phenomenon and the synthesis of different classes and subclasses of Ig in the mammary gland of cattle has been studied by various investigators (Mackenzie and Lascelles, 1968; Brandon et al., 1971; Butler et al., 1972; Morgan et al., 1981). Differences between Ig of bovine serum and colostrum or milk have also been investigated (Mukkur and Froese, 1971; Lisowski et al., 1975; Tewari and Mukkur, 1975; Gorczyca et al., 1986; Kulczycki et al., 1987). Fragments of Ig have also been investigated, including those of bovine colostrum IgG1 (Wie et al., 1978), and

other bovine Ig (Godger, 1971; Beh, 1973; Butler, 1973). Briefly, IgG, and in particular IgG1, is the major Ig found in bovine colostrum and milk (Dixon et al., 1961; Brandon et al., 1971; Butler et al., 1972). The preferential occurrence of a single IgG subclass in lacteal secretion suggests a subclass-specific transport mechanism. While many have observed the phenomenon (Mackenzie and Lascelles, 1968; Brandon et al., 1971; Butler et al., 1972; Morgan et al., 1981), the mechanism is still unclear. Early studies suggested an Fc-specific transport receptor on the acinar epithelial cells of the mammary gland (Kemler et al., 1975). More recently, Kaskovics et al. (2000) have described a homolog of FcRn from the bovine mammary gland. A homolog of this receptor is responsible for IgG transport across the rat gut epithelial cells. Changes in the cellular localization of this receptor in the epithelial cells of the ruminant mammary gland during times of rapid transport vs. reduced transport suggest that bovine FcRn might have an important role in the transport of bovine IgG1 across the acinar epithelial cells of the bovine mammary gland.

Immunoglobulin G1 is not the only IgG found in bovine milk and colostrum. The existence of 2 major subclasses of bovine IgG, namely IgG1 and IgG2, has been known for >3 decades, and the existence of a third subclass has been proposed (Kickhofen et al., 1968). Over the years, these have endured various changes in nomenclature, e.g., a) IgG2a, IgG2b, and IgG1 (Butler et al., 1987, 1994); b) bovine γ 1, γ 2, and γ 3 (Knight and Becker, 1987; Knight et al., 1988); and c) γ 2, γ 1, and γ Gs (Hammer et al., 1968; Kickhofen et al., 1968; Mossman et al., 1973). The nomenclature of the Kickhofen and Mossman group is no longer used. The description of IgG2b or IgG3 was based on serological (Butler et al., 1987) and genetic studies (Knight et al., 1988), and it was speculated that the 2 are in fact identical (Heyerman et al., 1992). The work of Rabbani et al. (1997) at the Karolinska Institute has clearly confirmed the existence of 3 IgG subclasses in cattle, and, by international agreement, these are now recognized as IgG1, IgG2, and IgG3 (Table 5).

The relative proportions of IgG1 and IgG2 differ in serum and mammary secretions; whereas serum contains nearly equivalent proportions of IgG1 and IgG2, the ratio of IgG1:IgG2 in colostrum and mature milk is 15 to 20:1 and 4 to 7:1, respectively (Guidry et al., 1980). As historically shown, IgG1 is selectively transported by the mammary gland (see previous), although during inflammation (mastitis), there is substantial transudation into milk of all serum proteins including IgG2 (Mackenzie and Lascelles, 1968; Butler et al., 1972; Butler and Heyerman, 1986; Hidirolglou et al., 1992). Very little is known about the distribution and

Table 6. The Ig in lacteal secretions of representative species.¹

Species	Ig	Concentration (mg/mL)		
		Colostrum	Mature milk	Serum
Human	IgG (total)	0.85 ± 0.21	0.04	12.4
	IgA	86.8 ± 13.6	1	2.91
	IgM	2.64 ± 0.92	0.1	1.17
Rat	IgG (total)	2.6	1.06	24.6
	IgG2a	0.67	0.8	6.91
	IgG2b	NA	0.26	0.89
	IgA	1.15	1.02	0.18
	IgM	ND	>0.002	0.95
Rabbit	IgG	1.5	0.1	5 →10
	IgA	~30.0	~5.0	0.01
	IgM	0.01	Trace	0.01
Dog	IgG (total)	12.1	0.15	15.6
	IgA	3.6	1.75	0.94
	IgM	0.6	0.13	1.6
Swine	IgG (total)	61.8	1.6	24
	IgA	11.3	4.1	2
	IgM	3.8	1.5	2.5
Cattle	IgG1	46.4	0.58	11.2
	IgG2	2.87	0.05	9.2
	IgG3	NA	NA	NA
	IgA	5.36	0.1	0.37
	IgM	6.77	0.09	3.1
Sheep	IgG1	94 →162	1	18.1
	IgG2	2	0.1	7.9
	IgA	3.5	0.2	0.2
	IgM	1.3→21.2	0.2	3.6
Horse	IgG1/IgG2 (IgGa)	82	0.2	3.4*
	IgG4/IgG5 (IgGb)	183	0.3	19.6
	? (IgGc)	0.3	ND	0.2
	IgG3 [IgG(T)]	44	0.1	4
	IgA	9	0.7	0.4
	IgM	2.3	0.07	1.6
Cat	IgG	26.5	ND	14.7
	IgA	0.64	ND	2.2
	IgM	0.48	ND	5.3

¹Values for IgE and IgD are not given for any species, although in both of these, quantitatively minor Ig occur in trace amounts of IgE in milk and in colostrum of various species. Precise quantitative data are unavailable. All data are from Butler and Kehrli (2004). NA = not available; ND = not determined.

biological function of IgG3 as no IgG3-specific antibodies are currently available for such studies.

LF

A family of specific iron-binding proteins occurs in milk. The major bovine protein is LF, or lactotransferrin to distinguish it from the serum transferrin that also occurs in milk (Groves, 1960) particularly in colostrum (Groves, 1971). Lactoferrin is of mammary origin and can be found in the milk of most species (Schanbacher et al., 1993). However, LF also occurs in secretions of other epithelial cells (Masson et al., 1966), as well as in polymorphonuclear leucocytes (Baggiolini, 1972). The mammary reference protein occurs at a concentration between 20 and 200 µg/L. The actual concentration varies and increases noticeably in response to inflamma-

tion or infection. The family occurs as a single-chain polypeptide with varying degrees of glycosylation. It consists of 689 amino acids as follows: Asp₃₆, Asn₂₉, Thr₃₆, Ser₄₅, Glu₄₀, Gln₂₉, Pro₃₀, Gly₄₉, Ala₆₇, Cys₃₄, Val₄₆, Met₄, Ile₁₆, Leu₆₆, Tyr₂₁, Phe₂₇, Lys₅₄, His₁₀, Trp₁₃, Arg₃₇. Lactoferrin has a calculated molecular weight of 76,110, taking its 17 disulfide bonds into account. Measured molecular weights vary, depending primarily on the level of glycosylation (Hurley et al., 1993). The primary structure (Figure 12) was determined in the early 1990s (Mead and Tweedie, 1990; Goodman and Schanbacher, 1991; Pierce et al., 1991). The LF signal peptide is composed of 19 amino acids, making the pre-form of LF 708 amino acids in length. Lactoferrin is listed in the ExPASy database as TRFL_Bovin; its file number is P24627. The 3-D structure of bovine holo-LF at 2.8 Å determined by Moore et al. (1997) can be

```

10                                20
Ala-Pro-Arg-Lys-Asn-Val-Arg-Trp-Cys-Thr-Ile-Ser-Gln-Pro-Glu-Trp-Phe-Lys-Cys-Arg-
30                                40
Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-
50                                60
Phe-Ala-Leu-Glu-Cys-Ile-Arg-Ala-Ile-Ala-Glu-Lys-Lys-Ala-Asp-Ala-Val-Thr-Leu-Asp-
70                                80
Gly-Gly-Met-Val-Phe-Glu-Ala-Gly-Arg-Asp-Pro-Tyr-Lys-Leu-Arg-Pro-Val-Ala-Ala-Glu-
90                                100
Ile-Tyr-Gly-Thr-Lys-Glu-Ser-Pro-Gln-Thr-His-Tyr-Tyr-Ala-Val-Ala-Val-Val-Lys-Lys-
110                               120
Gly-Ser-Asn-Phe-Gln-Leu-Asp-Gln-Leu-Gln-Gly-Arg-Lys-Ser-Cys-His-Thr-Gly-Leu-Gly-
130                               140
Arg-Ser-Ala-Gly-Trp-Ile-Ile-Pro-Met-Gly-Ile-Leu-Arg-Pro-Tyr-Leu-Ser-Trp-Thr-Glu-
150                               160
Ser-Leu-Glu-Pro-Leu-Gln-Gly-Ala-Val-Ala-Lys-Phe-Phe-Ser-Ala-Ser-Cys-Val-Pro-Cys-
170                               180
Ile-Asp-Arg-Gln-Ala-Tyr-Pro-Asn-Leu-Cys-Gln-Leu-Cys-Lys-Gly-Glu-Gly-Glu-Asn-Gln-
190                               200
Cys-Ala-Cys-Ser-Ser-Arg-Glu-Pro-Tyr-Phe-Gly-Tyr-Ser-Gly-Ala-Phe-Lys-Cys-Leu-Gln-
210                               220
Asp-Gly-Ala-Gly-Asp-Val-Ala-Phe-Val-Lys-Glu-Thr-Thr-Val-Phe-Glu-Asn-Leu-Pro-Glu-
230                               240
Lys-Ala-Asp-Arg-Asp-Gln-Tyr-Glu-Leu-Leu-Cys-Leu-Asn-Asn-Ser-Arg-Ala-Pro-Val-Asp-
250                               260
Ala-Phe-Lys-Glu-Cys-His-Leu-Ala-Gln-Val-Pro-Ser-His-Ala-Val-Val-Ala-Arg-Ser-Val-
270                               280
Asp-Gly-Lys-Glu-Asp-Leu-Ile-Trp-Lys-Leu-Leu-Ser-Lys-Ala-Gln-Glu-Lys-Phe-Gly-Lys-
290                               300
Asn-Lys-Ser-Arg-Ser-Phe-Gln-Leu-Phe-Gly-Ser-Pro-Pro-Gly-Gln-Arg-Asp-Leu-Leu-Phe-
310                               320
Lys-Asp-Ser-Ala-Leu-Gly-Phe-Leu-Arg-Ile-Pro-Ser-Lys-Val-Asp-Ser-Ala-Leu-Tyr-Leu-
330                               340
Gly-Ser-Arg-Tyr-Leu-Thr-Thr-Leu-Lys-Asn-Leu-Arg-Glu-Thr-Ala-Glu-Glu-Val-Lys-Ala-
350                               360
Arg-Tyr-Thr-Arg-Val-Val-Trp-Cys-Ala-Val-Gly-Pro-Glu-Glu-Gln-Lys-Lys-Cys-Gln-Gln-
370                               380
Trp-Ser-Gln-Gln-Ser-Gly-Gln-Asn-Val-Thr-Cys-Ala-Thr-Ala-Ser-Thr-Thr-Asp-Asp-Cys-
390                               400
Ile-Val-Leu-Val-Leu-Lys-Gly-Glu-Ala-Asp-Ala-Leu-Asn-Leu-Asp-Gly-Gly-Tyr-Ile-Tyr-
410                               420
Thr-Ala-Gly-Lys-Cys-Gly-Leu-Val-Pro-Val-Leu-Ala-Glu-Asn-Arg-Lys-Ser-Ser-Lys-His-
430                               440
Ser-Ser-Leu-Asp-Cys-Val-Leu-Arg-Pro-Thr-Glu-Gly-Tyr-Leu-Ala-Val-Ala-Val-Val-Lys-
450                               460
Lys-Ala-Asn-Glu-Gly-Leu-Thr-Trp-Asn-Ser-Leu-Lys-Asp-Lys-Lys-Ser-Cys-His-Thr-Ala-
470                               480
Val-Asp-Arg-Thr-Ala-Gly-Trp-Asn-Ile-Pro-Met-Gly-Leu-Ile-Val-Asn-Gln-Thr-Gly-Ser-
490                               500
Cys-Ala-Phe-Asp-Glu-Phe-Phe-Ser-Gln-Ser-Cys-Ala-Pro-Gly-Ala-Asp-Pro-Lys-Ser-Arg-
510                               520
Leu-Cys-Ala-Leu-Cys-Ala-Gly-Asp-Asp-Gln-Gly-Leu-Asp-Lys-Cys-Val-Pro-Asn-Ser-Lys-
530                               540
Glu-Lys-Tyr-Tyr-Gly-Tyr-Thr-Gly-Ala-Phe-Arg-Cys-Leu-Ala-Glu-Asp-Val-Gly-Asp-Val-
550                               560
Ala-Phe-Val-Lys-Asn-Asp-Thr-Val-Trp-Glu-Asn-Thr-Asn-Gly-Glu-Ser-Thr-Ala-Asp-Trp-
570                               580
Ala-Lys-Asn-Leu-Asn-Arg-Glu-Asp-Phe-Arg-Leu-Leu-Cys-Leu-Asp-Gly-Thr-Arg-Lys-Pro-
590                               600
Val-Thr-Glu-Ala-Gln-Ser-Cys-His-Leu-Ala-Val-Ala-Pro-Asn-His-Ala-Val-Val-Ser-Arg-
610                               620
Ser-Asp-Arg-Ala-Ala-His-Val-Lys-Gln-Val-Leu-Leu-His-Gln-Gln-Ala-Leu-Phe-Gly-Lys-
630                               640
Asn-Gly-Lys-Asn-Cys-Pro-Asp-Lys-Phe-Cys-Leu-Phe-Lys-Ser-Glu-Thr-Lys-Asn-Leu-Leu-
650                               660
Phe-Asn-Asp-Asn-Thr-Glu-Cys-Leu-Ala-Lys-Leu-Gly-Gly-Arg-Pro-Thr-Tyr-Glu-Glu-Tyr-
670                               680
Leu-Gly-Thr-Glu-Tyr-Val-Thr-Ala-Ile-Ala-Asn-Leu-Lys-Lys-Cys-Ser-Thr-Ser-Pro-Leu-
689
Leu-Glu-Ala-Cys-Ala-Phe-Leu-Thr-Arg.OH

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Figure 12. Primary structure of *Bos lactoferrin* (LF). Disulfides occur between the following Cys: 9 and 45, 19 and 36, 115 and 198, 157 and 173, 160 and 183, 170 and 181, 231 and 245, 348 and 380, 358 and 371, 405 and 684, 425 and 647, 457 and 532, 481 and 675, 491 and 505, 502 and 515, 573 and 587, and 625 and 630. The 2 iron-binding sites include (Asp60, Tyr92, Tyr192, His253) and (Asp395, Tyr433, Tyr526, His595). The anion-binding sites are (Arg121, Thr117) and (Arg463, Thr459). The potential glycosylation sites are Asn233, Asn281, Asn368, Asn476, and Asn545. The PDB (protein structural database) file for this protein is 1BLF.

found in the Protein Data Bank with the identifier 1BLF (Berman et al., 2000). Lactoferrin consists of a single-polypeptide chain that folds into 2 globular lobes an N-lobe (residues 1 to 333) and a C-lobe (residues 345 to 676) connected by a 3-turn helix (residues 334 to 344). Each lobe is further divided into 2 domains (N1, residues 1 to 90 and 251 to 333, and N2, residues 91 to 250; C1, residues 345 to 431 and 593 to 676, and C2, residues 432 to 592). Bovine LF has 5 potential glycosylation sites (Figure 12) (Pierce et al., 1991), and recently Wei et al. (2000) reported glycans at each of the 5 sites. The composition of these glycans is complex and varies with the stage of lactation. At the time of its discovery, the reddish color and compositional similarity to transferrin and ovotransferrin suggested that LF might have a role in iron transport (Groves, 1960). Lactoferrin fully saturated with Fe^{+3} is called hololactoferrin and has a salmon color; in its native state, the degree of saturation is in the range of 15 to 40%. Iron-depleted LF is known as apolactoferrin. Early studies (Brown and Parry, 1974; Parry and Brown, 1974) focused on the conformational effects of iron binding as well as identification of side chain ligands. In the detailed description (Moore et al., 1997), each iron-binding site is located in a cleft between the 2 domains of a lobe. The anion, carbonate, or bicarbonate, required for iron binding, occupies a positively charged pocket in the wall of the N2 or C2 domain (Moore et al., 1997). Because the iron-LF complex is very stable, iron is released only below pH 3.5, whereas iron release by transferrin occurs in mildly acidic media of pH 5.5 (Abdallah and El Hage Chahine, 2000); it may more properly be classed as an iron scavenger than an iron transport protein.

Biological Function

The role of LF as a first line defense against infection and inflammation was recently reviewed by Ward et al. (2002). The earliest recognized biological function of LF was its role in bacteriostasis, because of the ability to sequester iron from a relatively iron-free environment, thus removing an essential nutrient for bacteria (Smith and Schanbacher, 1977; Bullen et al., 1978). Subsequent research has identified a multitude of possible functions that may or may not involve iron binding. Among these is an antibacterial activity caused by the ability of apo-LF to bind the outer membrane of Gram-negative bacteria, causing the rapid release of lipopolysaccharides and an increase in membrane permeability (Arnold et al., 1977). This activity has been localized to a pepsin-cleavable active peptide from the N-terminal domain, not related to iron binding (Vogel et al., 2002). Lactoferrin also displays antiviral activity against both

DNA and RNA viruses; the published mechanisms of the antiviral functions of LF are comprehensively reviewed by Van der Strate et al (2001).

Technological Function

Because protein structure dictates function, it should not be surprising that a protein with the complex structure of LF would prove to be multifunctional. Also, not surprising is the attractiveness of such a multifunctional protein for commercial development. Despite the low concentration of LF in bovine milk, it is now being isolated and purified on an industrial scale from cheese whey, mainly in Japan and Europe (Horton, 1995). Among its useful characteristics, the heat stability of LF is such that typical pasteurization processes have little effect on structure (Sanchez et al., 1992). Development is beginning on commercial applications utilizing bovine LF and its partially digested peptides as nutraceuticals in infant formulas, health supplements, oral care products, and animal feeds to capitalize on its ability to boost natural defense against infections (Sanchez et al., 1992). Its potential for use as an antioxidant is beginning to see realization in cosmetics (Steinjns and van Hooijdonk, 2000). The commercial production of LF has given impetus to research on lactoferricin(s), 15-residue peptides in pepsin hydrolysates of LF (Strom et al., 2000), which have antimicrobial activity of their own (Strom et al., 2002).

Over the past decade, LF has become that rare protein for which there is comprehensive structural data and a multitude of functions. In recognition of this, 5 biennial conferences dealing solely with lactoferrin have now been held, most recently in 2001 (Conference Abstracts, 2002).

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